

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant :	Bonastre et al.	Art Unit :	1633
Patent No. :	7,344,711	Examiner :	Scott D. Priebe
Issue Date :	March 18, 2008	Conf. No. :	7846
Serial No. :	10/509,194		
Filed :	September 23, 2004		
Title :	USE OF ADENOVIRUSES MUTATED IN THE VA GENES FOR CANCER TREATMENT		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPLICATION FOR PATENT TERM ADJUSTMENT UNDER 37 CFR §1.705(D)

Applicants hereby petition for reconsideration of the Patent Term Adjustment (PTA) accorded the above-referenced patent. Attached herewith is a copy of the Issue Notification including a Determination of Patent Term Adjustment under 35 U.S.C. 154(b), mailed February 27, 2008, for the above-referenced patent. The notification, as well as the face of the patent, states that the Patent Term Adjustment at issuance is 346 days. Reconsideration of the Patent Term Adjustment to decrease Applicant Delay from 58 days to 50 days, to increase PTO delay from 404 days to 570 days, and to increase Total PTA from 346 days to 520 days, is respectfully requested.

REVIEW OF PATENT TERM ADJUSTMENT CALCULATION

A review of the Patent Term Adjustment History in the PAIR system shows that the United States Patent and Trademark Office (PTO) calculated the Patent Term Adjustment (PTA) as follows:

- 1) The PTO mailed a delayed 14-month first non-final Office Action on December 21, 2006. Applicant agrees with the calculated PTO Delay of 393 days.
- 2) Applicants submitted a response to the above-referenced non-final Office Action on March 21, 2007; however this was deemed informal or non-responsive by the PTO. Applicants submitted a proper response on May 10, 2007, which the PTO received on the same date. Applicants agree with the calculated Applicant Delay of 50 days.
- 3) The PTO mailed a Notice of Allowance on September 14, 2007. On October 24, 2007, prior to payment of the issue fee, Applicants filed via EFS-Web a Request for Consideration Under 37 CFR §1.8 for the purpose of resubmitting an Information Disclosure Statement originally filed on November 2, 2005 but not entered into the file or considered by the Examiner. A copy of this electronic filing with Electronic Acknowledgement Receipt is attached hereto. In the PAIR Patent Term Adjustment History under date of October 24, 2007, the document is entered as an "Information Disclosure Statement." Applicants respectfully disagree with the calculated Applicant Delay of 8 days and hereby request its removal.
- 4) Applicant paid the issue fee electronically on November 7, 2007 and it was received by the PTO on the same date. The patent did not grant until March 18, 2007. Applicant agrees with the PTO Delay of 11 days in granting the patent; however Applicant believes this is inapplicable in light of the delay in issuance of a patent not more than three years from the filing date under 35 USC §154(b)(1)(B) which is not included in the PTO's calculation. The patent

Applicant : Bonastre et al.
Patent No. : 7,344,711
Issued : March 18, 2008
Serial No. : 10/509,194
Filed : September 23, 2004
Page : 3 of 4

Attorney's Docket No.: 16596-049US1

in this matter should have issued no later than September 23, 2007 and did not issue until March 18, 2008, resulting in PTO Delay of 177 days instead of 11 days. Applicant respectfully requests that the PTO Delay of 11 days for grant of the patent more than four months after payment of the issue fee be increased to 177 days for grant of the patent more than three years after the filing date, resulting in a net increase in PTO Delay of 166 days.

REMARKS

In consideration of the events described above, Applicants believe the PTA calculation of 346 days is incorrect.

Pursuant to 37 CFR §1.704(c)(10) or 1247 OG 111 (Notice dated June 26, 2001), the filing of a Request for Consideration Under 37 CFR §1.8 does not cause interferences or delay and will not be considered a "failure to engage in reasonable efforts" to conclude processing or examination of an application and will not result in reduction of a patent term adjustment. Therefore, Applicants respectfully request the removal of 8 days of Applicant Delay and the addition of 166 days of PTO Delay, thus decreasing Applicant Delay from 58 days to 50 days, increasing the PTO Delay from 404 days to 570 days, and increasing the Total PTA from 346 days to 520 days.

A copy of the Request for Consideration Under 37 CFR §1.8 filed electronically on October 24, 2007 together with its Electronic Acknowledgement Receipt is provided herein.

The fee of \$200 required under 37 CFR §1.18(e) is being paid concurrently herewith on the Electronic Filing System (EFS) by way of Deposit Account authorization. Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: March 19, 2008



Tina Williams McKeon
Reg. No. 43,791

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UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/509,194	03/18/2008	7344711	16596-049US1	7846

26176 7590 02/27/2008
FISH & RICHARDSON P.C.
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MINNEAPOLIS, MN 55440-1022

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 346 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

Ramon Alemany Bonastre, Barcelona, SPAIN;
Manel Maria Cascallo Piqueras, Barcelona, SPAIN;

COPY

Electronic Acknowledgement Receipt

EFS ID:	2362348
Application Number:	10509194
International Application Number:	
Confirmation Number:	7846
Title of Invention:	USE OF ADENOVIRUSES MUTATED IN THE VA GENES FOR CANCER TREATMENT
First Named Inventor/Applicant Name:	Ramon Alemany Bonastre
Customer Number:	26176
Filer:	Janice Kugler DeYoung/Susan Brandt
Filer Authorized By:	Janice Kugler DeYoung
Attorney Docket Number:	16596-049US1
Receipt Date:	24-OCT-2007
Filing Date:	23-SEP-2004
Time Stamp:	14:03:10
Application Type:	U.S. National Stage under 35 USC 371

COPY

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes) /Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Arguments/Remarks Made in an Amendment	16596049US1ResubmitIDS.pdf	506996 5949ce04ed6631037b515bc7cce0697a8af0faab	no	13

Warnings:

Information:					
2	Foreign Reference	WO1999057296A1.pdf	2244808	no	50
			6da9bd662c7d9b2829f3a81f289e590e 6ab06700		
Warnings:					
Information:					
Total Files Size (in bytes):				2751804	
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p><u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p><u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p><u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>					

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Bonastre et al. Art Unit : 1616
Serial No. : 10/509,194 Examiner : Scott D. Priebe
Filed : September 23, 2004 Conf. No. : 7846
Title : USE OF ADENOVIRUSES MUTATED IN THE VA GENES FOR CANCER
TREATMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CONSIDERATION UNDER 37 CFR 1.8

Applicants hereby request consideration of the Information Disclosure Statement, Form PTO-1449, and the references cited therein, which were submitted by applicants on November 2, 2005, via Express Mail. The submission was apparently not entered into the official file, as it appears in neither the PAIR system nor the PALM system, as confirmed by Examiner Priebe.

Applicants submit herewith a copy of that submission, a copy of a return receipt postcard indicating that the papers were received in the Patent Office, as well as a copy of a Declaration of Mailing by Express Mail indicating that the submission was deposited on November 2, 2005.

Applicants believe that no fees are due, as the submission was timely filed before the first office action on the merits, which was not mailed until December 21, 2006. However, please apply any necessary charges or credits to Deposit Account No. 06-1050, referencing Attorney Docket No. 16596-049US1.

Respectfully submitted,

Date:

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21767889.doc

Janice Kugler DeYoung
Reg. No. 50,429

COPY

CERTIFICATE OF MAILING BY EFS-WEB FILING

I hereby certify that this paper was filed with the Patent and Trademark Office using the EFS-WEB system on this date: October 24, 2007

Substitute Form PTO-1449 (Modified)	U.S. Department of Commerce Patent and Trademark Office	Attorney's Docket No. 16596-049US1	Application No. 10/509,194
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))		Applicant Alemany Bonastre, Ramon, et al.	
		Filing Date September 23, 2004	Group Art Unit Unknown

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	AA	5981225	11/1999	Kochanek et al.			
	AB						
	AC						
	AD						
	AE						
	AF						
	AG						
	AH						
	AI						
	AJ						
	AK						

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	AL	99/57296	11/1999	PCT				
	AM							
	AN							
	AO							
	AP							

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	AQ	
	AR	
	AS	
	AT	

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	



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☎ (070) 3 40 20 40
FAX (070) 3 40 30 16

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Patentamt

European
Patent Office

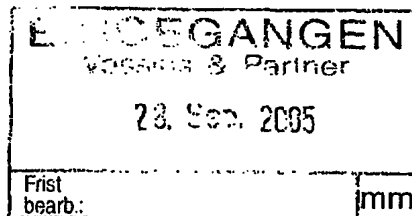
Office européen
des brevets

Generaldirektion 1

Directorate General 1

Direction générale 1

Vossius & Partner
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81675 München
ALLEMAGNE



EPO Customer Services

Tel.: +31 (0)70 340 45 00

Date

27.09.05

Reference K2367 EP/1S3	Application No./Patent No. 05016560.4 - 2405 PCT/
Applicant/Proprietor Oncolytics Biotech Inc.	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 44a EPC, the European search report (R. 44 EPC) or the partial European search report/ declaration of no search (R. 45 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☒ 1 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☒ Abstract

☒ Title

☐ the Abstract was modified and the definitive text is attached to this communication.

The following figure will be published together with the abstract: 0

Refund of the search fee

If applicable under Article 10 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.





DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 01/35970 A (ONCOLYTICS BIOTECH INC; COFFEY, MATTHEW, C; THOMPSON, BRADLEY, G) 25 May 2001 (2001-05-25)	1-6,9,11	C12N15/861 C12N7/04 A61P35/00 A61K35/76
Y	* the whole document *	7,8,10	
Y	WO 98/35028 A (BAXTER INTERNATIONAL INC) 13 August 1998 (1998-08-13)	7,8,10	
	* page 8, lines 15-32 *		
A	* page 18, line 20 - page 26, line 2 *	4,9,11	
A	US 5 002 874 A (KAUFMAN ET AL) 26 March 1991 (1991-03-26)	1-11	
	* the whole document *		
X	US 5 981 225 A (KOCHANNEK ET AL) 9 November 1999 (1999-11-09)	6-11	
Y	* claims 17-28 *	1-5	
	* column 9, line 60 - column 11, line 3 *		
X	WO 99/57296 A (GENZYME CORPORATION; WADSWORTH, SAMUEL, C; SCARIA, ABRAHAM) 11 November 1999 (1999-11-11)	6-11	
Y	* claims 1-20 *	1-5	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
			C12N A61K A61P
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 19 September 2005	Examiner Fayos, C
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons	

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 05 01 6560

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

19-09-2005

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0135970	A	25-05-2001	AU 782020 B2	30-06-2005
			AU 1262601 A	30-05-2001
			BR 0015491 A	15-10-2002
			CA 2388807 A1	25-05-2001
			EP 1227828 A1	07-08-2002
			JP 2003514024 T	15-04-2003
			MX PA02004736 A	28-01-2003
			NZ 518454 A	30-07-2004
			ZA 200203041 A	09-12-2003
WO 9835028	A	13-08-1998	CA 2280237 A1	13-08-1998
			EP 0968281 A2	05-01-2000
			JP 2001511012 T	07-08-2001
			US 2002142989 A1	03-10-2002
			US 6403370 B1	11-06-2002
US 5002874	A	26-03-1991	NONE	
US 5981225	A	09-11-1999	AU 3635899 A	01-11-1999
			WO 9953089 A1	21-10-1999
WO 9957296	A	11-11-1999	NONE	

**Bescheld/Protokoll (Anlage)**

Datum
Date cf Form 1507
Date

Communication/Minutes (Annex)

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Sheet 1
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Notification/Procès-verbal (Annexe)

Anmelde-Nr.:
Application No.: 05 016 560.4
Demande n°:

The examination is being carried out on the **following application documents**:

Description, Pages

1-24 as originally filed

Claims, Numbers

1-11 as originally filed

Drawings, Sheets

1/6-6/6 as originally filed

- 1- Patent application 05016560.4 (referred to herein and for the rest of the procedure as present application) is a divisional application of patent application 03744873.5 (referred to herein and for the rest of the procedure as parent application).
- 2- The parent application has been examined according to the PCT and to the EPC with a communication according to Rule 51(4) EPC being issued for the sole patentable subject matter.

Now the applicant has filed claims 1-11 which are those originally filed for the parent application, as a divisional application.

During the examination of the parent application it was made clear (also by means of a telephone conversation) that the sole patentable subject matter present in the application is that which has already been the subject of a grant for the parent application.

**Bescheid/Protokoll (Anlage)****Communication/Minutes (Annex)****Notification/Procès-verbal (Annexe)**

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Date
Date
cf Form 1507

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2

Anmelde-Nr.:
Application No.: 05 016 560.4
Demande n°:

It is an accepted principle in most patent systems that two or more patents shall not be granted to the same applicant for one invention. The applicant should amend on or more of the applications claiming the same subject matter (the same bacterial strain) in such a manner that they no longer claim the same invention, or choose which one of those applications he wishes to grant (see Guidelines C-IV 6.4). T118/91, T441/92 T587/98.

PRIOR ART

- 3- Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure:
- D1: WO 01/35970 A (ONCOLYTICS BIOTECH INC; COFFEY, MATTHEW, C; THOMPSON, BRADLEY, G) 25 May 2001 (2001-05-25)
- D2: WO 98/35028 A (BAXTER INTERNATIONAL INC) 13 August 1998 (1998-08-13)
- D3: US-A-5 002 874 (KAUFMAN ET AL) 26 March 1991 (1991-03-26)
- D4: US-A-5 981 225 (KOCHANNEK ET AL) 9 November 1999 (1999-11-09)
- D5: WO 99/57296 A (GENZYME CORPORATION; WADSWORTH, SAMUEL, C; SCARIA, ABRAHAM) 11 November 1999 (1999-11-11)

NOVELTY - Art. 54 EPC

- 4- D1 claims the use of modified adenovirus for the treatment of a Ras-mediated cell proliferative disorder in a mammal having a Ras-activated pathway (see claim 1), in which the modified adenovirus lacks the gene encoding VAI RNA (see claim 2 and examples).

Present independent claim 1 is however directed to the use of an adenovirus defective in its VAI and VAII virus-associated RNAs. Hence, D1 does not appear to be novelty destroying for the subject matter of present claims 1-5, but is novelty destroying for the subject matter of claims 6 and 9-11.

D2 relates to a complementary-Ad vector system for killing tumor cells and mentions E1 deletion or substitution by the replacement of the E1a promoter by one which is active mainly in hepatocarcinoma cells or by deletion or mutation of E1b-p55 or E1b (see also



Bescheid/Protokoll (Anlage)

Communication/Minutes (Annex)

Notification/Procès-verbal (Annexe)

Datum
Date
Date

cf Form 1507

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3

Anmelde-Nr.:
Application No.: 05 016 560.4
Demande n°:

the claims).

Hence, D2 destroys the novelty of claims 6-11.

The applicant's attention is drawn to the fact that a composition is only defined by its components and not by its intended use or alleged effects. Therefore, D4 or D5 destroy the novelty of claims 6-11.

INVENTIVE STEP - Art. 56 EPC

5- The closest prior art is represented by D1.

The closest prior art differs from the present application in that it does not mention a further defect / mutation in VAI.

The technical effect achieved in the present application is the provision of a better (more selective) cancer treatment.

The objective problem posed in the present application is to provide further adenovirus mutants for the purpose of treating cancer.

The solution proposed is an adenovirus defective in its VAI and VAI RNAs.

No technical evidence has been provided in the present application showing that an adenovirus with both VAI and VAI RNAs mutated is more selective for tumor cells with an active Ras pathway than an adenovirus with only the VAI RNA mutated.

It is to be noted that the examples of the present application relate to a VAI RNA mutant (dl331) (see e.g. p 20 line 26) and do not mention at all a further defect / mutation on the VAI RNA (see also example 3 p 24).

On one hand, it is nowhere suggested in D1 alone, or taken in combination with D2 to provide an adenovirus defective in its VAI and VAI virus-associated RNAs. On the other hand, this combination of features is nowhere shown to provide a technical effect or advantage over what is already known from the prior art at hand.

Case of claims 6-11 appear to be neither novel, nor inventive, since no technical effect, other than what is already achieved in D1 has been demonstrated.

**Bescheld/Protokoll (Anlage)****Communication/Minutes (Annex)****Notification/Procès-verbal (Annexe)**Datum
Date
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4

Anmelde-Nr.:
Application No.: 05 016 560.4
Demande n°:

A technical effect solving a technical problem has to be achieved by all embodiments falling within the scope of the claim. Claims covering embodiments not achieving said effect, and thus not solving the underlying technical problem, do not meet the requirements of Art. 56 EPC, for everything falling within a valid claim has to be inventive (in line with T939/92).

Hence, it seems that claims 1-11 lack inventive step.

FINAL REMARKS

- 6- In view of the above, and in particular of item 1-, it is not at present apparent which part of the application could serve as a basis for a new, allowable claim. Should the applicant nevertheless regard some particular matter as patentable, an independent claim should be filed taking account of Rule 29(1) EPC. The applicant should also indicate the difference of the subject-matter of the new claim vis-à-vis the state of the art and the significance thereof.



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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Alemany Bonastre, Ramon, et al.
Serial No.: 10/509,194
Filed: September 23, 2004
Title: USE OF ADENOVIRUSES MUTATED IN THE VA GENES FOR CANCER
TREATMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MAILING BY "EXPRESS MAIL"

Patty Bobay declares as follows:

1. I reside at:

EV584753054US

#1 Whittier Street
Milpitas, CA 95035

and am an employee of:

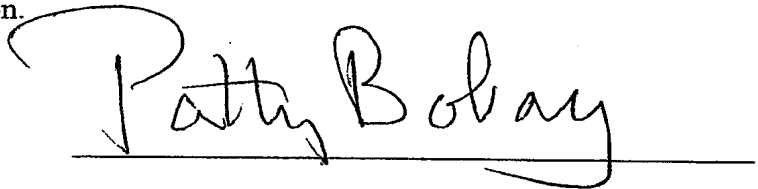
Fish & Richardson P.C.
500 Arguello Street, Suite 500
Redwood City, California 94063

2. On November 2, 2005, I deposited in the mail, "Express Mail Post Office to Addressee" service of the United States Postal Service, the contents of the envelope for which "Express Mail" receipt No. EV 584753054 US was issued addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

3. Attached hereto is a true copy of the "Express Mail" receipt No. EV 584753054 US.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patents issued thereon.

Date: November 2, 2005

A handwritten signature in cursive script that reads "Patty Bobay". The signature is written in dark ink and is positioned above a horizontal line.

16596-049US1

EV 584753054 US

Nov. 2, 2005

10/509,194

September 23, 2004

MAD/mum/myc

USE OF ADENOVIRUSES MUTATED IN THE VA GENES FOR CANCER TREATMENT

Alemaný Bonastre, Ramon, et al.

Information Disclosure Statement (1 page)

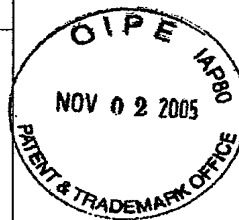
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(54) Title: PARTIALLY DELETED ADENOVIRAL VECTORS			
(57) Abstract			
<p>The invention is directed to novel partially deleted adenoviral vectors (DeAd) in which the majority of adenoviral early genes required for replication are deleted from the vector and placed within the chromosome of a producer cell line under conditional promoter control. The partially deleted adenoviral (DeAd) vectors of the invention can accommodate inserts, such as transgenes, of up to 12-15 kb in size. The invention is further directed to DeAd vector producer cell lines that contain the adenoviral early genes necessary for replication under conditional promoter control that allow for large scale production of vectors. The invention is also directed to methods for the production of DeAd vectors in such cell lines and to the use of such vectors to deliver transgenes to target cells.</p>			

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Partially Deleted Adenoviral Vectors

Description

The present application is a continuation in part of U.S. Patent Application Serial No. 60/118,118 filed February 1, 1999 which, in turn, is a continuation-in-part of U.S. Patent Application Serial No. 60/083,841 filed May 1, 1998, the disclosures of which are incorporated herein by reference.

Introduction

The invention is directed to novel partially deleted adenoviral (DeAd) vectors in which the majority of adenoviral early genes required for viral replication are deleted from the vector and placed within the chromosome of a producer cell line under the control of a conditional promoter. The partially deleted adenoviral (DeAd) vectors of the invention can accommodate inserts of foreign nucleic acids (transgenes) of up to 12-15 kb in size. The invention is further directed to DeAd vector producer cell lines that contain the adenovirus early genes necessary for viral replication under conditional promoter control that allow for large scale production of vectors. The invention is also directed to methods for the production of DeAd vectors in such cell lines and to the use of such vectors. The invention is still further directed to DeAd vectors in which the expression of the genes encoding virion structural proteins is made conditional by replacement of the major late promoter (MLP) with alternative promoters that can be controlled. Moreover, the invention is directed to DeAd vectors in which expression of the genes encoding virion structural proteins is diminished by deleting VA RNA genes from the vector.

Background of the Invention

Adenoviral vectors for use in gene transfer to cells and especially in gene therapy applications, commonly are derived from adenoviruses by deletion of the early region 1 (E1) genes (Berkner, K.L., *Curr. Top. Micro. Immunol.* 158:39-66, 1992). Deletion of E1 genes renders such adenoviral vectors replication defective and

significantly reduces expression of the remaining viral genes present within the vector. However, it is believed that the presence of the remaining viral genes in adenoviral vectors can be deleterious to the transfected cell for one or more of the following reasons: (1) stimulation of a cellular immune response directed against
5 expressed viral proteins, (2) cytotoxicity of expressed viral proteins, and (3) replication of the vector genome leading to cell death.

One solution to this problem has been vectors, which are adenoviral vectors derived from the genome of an adenovirus containing minimal *cis*-acting nucleotide sequences required for the replication and packaging of the vector genome and which
10 can contain one or more transgenes (*See*, U.S. Patent No. 5,882,877 which covers PAV vectors and methods for producing PAV, incorporated herein by reference). Such PAV vectors, which can accommodate up to 36 kb of foreign nucleic acid, are advantageous because the carrying capacity of the vector is optimized, while the potential for host immune responses to the vector or the generation of replication-
15 competent viruses is reduced. PAV vectors contain the 5' inverted terminal repeat (ITR) and the 3' ITR nucleotide sequences that contain the origin of replication, and the *cis*-acting nucleotide sequence required for packaging of the PAV genome, and can accommodate one or more transgenes with appropriate regulatory elements.

Adenoviral vectors, including PAV, have been designed to take advantage of
20 the desirable features of adenovirus which render it a suitable vehicle for nucleic acid transfer to recipient cells. Adenovirus is a non-enveloped, nuclear DNA virus with a genome size of about 36 kb, which has been well-characterized through studies in classical genetics and molecular biology (Horwitz, M.S., "Adenoviridae and Their Replication," in *Virology*, 2nd edition, Fields et al., eds., Raven Press, New York,
25 1990). The viral genes are classified into early (designated E1-E4) and late (designated L1-L5) transcriptional units, referring to the generation of two temporal classes of viral proteins. The demarcation between these events is viral DNA replication. The human adenoviruses are divided into numerous serotypes (approximately 47, numbered accordingly and classified into 6 subgroups: A, B, C, D,
30 E and F), based upon properties including hemagglutination of red blood cells,

oncogenicity, DNA base and protein amino acid compositions and homologies, and antigenic relationships.

Recombinant adenoviral vectors have several advantages for use as gene transfer vectors, including tropism for both dividing and non-dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector stocks, and the potential to carry large inserts (Berkner, K.L., *Curr. Top. Micro. Immunol.* 158:39-66, 1992; Jolly, D., *Cancer Gene Therapy* 1:51-64, 1994).

The cloning capacity of an adenovirus vector is proportional to the size of the adenovirus genome present in the vector. For example, a cloning capacity of about 8 kb can be created from the deletion of certain regions of the virus genome dispensable for virus growth, *e.g.*, E3, and the deletion of a genomic region such as E1 whose function may be restored *in trans* from 293 cells (Graham, F.L., *J. Gen. Virol.* 36:59-72, 1977) or A549 cells (Imler et al., *Gene Therapy* 3:75-84, 1996). Such E1-deleted vectors are rendered replication-defective. The upper limit of vector DNA capacity for optimal carrying capacity is about 105%-108% of the length of the wild-type genome. Further adenovirus genomic modifications are possible in vector design using cell lines which supply other viral gene products *in trans*, *e.g.*, complementation of E2a (Zhou et al., *J. Virol.* 70:7030-7038, 1996), complementation of E4 (Krougliak et al., *Hum. Gene Ther.* 6:1575-1586, 1995; Wang et al., *Gene Ther.* 2:775-783, 1995), or complementation of protein IX (Caravokyri et al., *J. Virol.* 69:6627-6633, 1995; Krougliak et al., *Hum. Gene Ther.* 6:1575-1586, 1995).

Maximal carrying capacity can be achieved using adenoviral vectors deleted for most viral coding sequences, including PAVs (U.S. Patent No. 5,882,877; Kochanek et al., *Proc. Natl. Acad. Sci. USA* 93:5731-5736, 1996; Parks et al., *Proc. Natl. Acad. Sci. USA* 93:13565-13570, 1996; Lieber et al., *J. Virol.* 70:8944-8960, 1996; Fisher et al., *Virology* 217:11-22, 1996; PCT Publication No. WO96/33280, published October 24, 1996; PCT Publication No. WO96/40955, published December 19, 1996; PCT Publication No. WO97/25446, published July 19, 1997; PCT Publication No. WO95/29993, published November 9, 1995; PCT Publication No.

WO96/13597, published May 9, 1996; PCT Publication No. WO97/00326, published January 3, 1997).

As noted above, PAV vectors can accommodate up to 36kb of foreign nucleic acid (U.S. Patent No. 5,882,877). Transgenes that have been expressed to date by
5 adenoviral vectors include *inter alia* p53 (Wills et al., *Human Gene Therapy* 5:1079-188, 1994); dystrophin (Vincent et al., *Nature Genetics* 5:130-134, 1993); erythropoietin (Descamps et al., *Human Gene Therapy* 5:979-985, 1994); ornithine transcarbamylase (Stratford-Perricaudet et al., *Human Gene Therapy* 1:241-256, 1990; We et al., *J. Biol. Chem.* 271:3639-3646, 1996); adenosine deaminase (Mitani
10 et al., *Human Gene Therapy* 5:941-948, 1994); interleukin-2 (Haddada et al., *Human Gene Therapy* 4:703-711, 1993); α 1-antitrypsin (Jaffe et al., *Nature Genetics* 1:372-378, 1992); thrombopoietin (Ohwada et al., *Blood* 88:778-784, 1996) and cytosine deaminase (Ohwada et al., *Hum. Gene Ther.* 7:1567-1576, 1996).

The tropism of adenoviruses for cells of the respiratory tract has particular
15 relevance to the use of adenoviral vectors in gene transfer for cystic fibrosis (CF), which is the most common autosomal recessive disease in Caucasians. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that disturb the cAMP-regulated Cl⁻ channel in airway epithelia result in pulmonary dysfunction (Zabner et al., *Nature Genetics* 6:75-83, 1994). Adenoviral vectors engineered to
20 carry the CFTR gene have been developed (Rich et al., *Human Gene Therapy* 4:461-476, 1993) and studies have shown the ability of these vectors to deliver CFTR to nasal epithelia of CF patients (Zabner et al., *Cell* 75:207-216, 1993), the airway epithelia of cotton rats and primates (Zabner et al., *Nature Genetics* 6:75-83, 1994), and the respiratory epithelium of CF patients (Crystal et al., *Nature Genetics* 8:42-51,
25 1994). Studies have shown that administering an adenoviral vector containing DNA encoding CFTR to airway epithelial cells of CF patients can restore a functioning chloride ion channel in the treated epithelial cells (Zabner et al., *J. Clin. Invest.* 97:1504-1511, 1996; U.S. Patent No. 5,670,488, issued September 23, 1997).

The use of adenoviral vectors in gene transfer studies to date indicates that
30 persistence of transgene expression in target cells and tissues is often transient. At ;

At least some of the limitation is due to the generation of a cellular immune response to the viral proteins which are expressed antigenically even from a replication-defective vector, triggering a pathological inflammatory response which may destroy or adversely affect the adenovirus-infected cells (Yang et al., *J. Virol.* 69:2004-2015, 1995; Yang et al., *Proc. Natl. Acad. Sci. USA* 91:4407-4411, 1994; Zsella et al., *Hum Gene Ther.* 6:457-467, 1995; Worgall et al., *Hum. Gene Ther.* 8:37-44, 1997; Kaplan et al., *Hum. Gene Ther.* 8:45-56, 1997). Because adenovirus does not integrate into the cell genome, host immune responses that destroy virions or infected cells have the potential to limit adenovirus-based gene transfer. An adverse immune response poses a serious obstacle for high dose administration of an adenoviral vector or for repeated administration (Crystal, R., *Science* 270:404-410, 1995).

In order to circumvent the host immune response, which limits the persistence of transgene expression, various strategies have been employed, that generally involve either the modulation of the immune response itself or the engineering of a vector that decreases the immune response. The administration of immunosuppressive agents, together with vector administration, has been shown to prolong transgene persistence (Fang et al., *Hum. Gene Ther.* 6:1039-1044, 1995; Kay et al., *Nature Genetics* 11:191-197, 1995; Zsella et al., *Hum. Gene Ther.* 6:457-467, 1995; Scaria et al., *Gene Therapy* 4:611-617, 1997; WO98/08541).

Modifications to genomic adenoviral sequences contained in the recombinant vector have been attempted in order to decrease the host immune response (Yang et al., *Nature Genetics* 7:362-369, 1994; Lieber et al., *J. Virol.* 70:8944-8960, 1996; Gorziglia et al., *J. Virol.* 70:4173-4178; Kochanek et al., *Proc. Natl. Acad. Sci. USA* 93:5731-5736, 1996; Fisher et al., *Virology* 217:11-22, 1996). The adenovirus E3 gp19K protein can complex with MHC Class I antigens and retain them in the endoplasmic reticulum, which prevents cell surface presentation and killing of infected cells by cytotoxic T-lymphocytes (CTLs) (Wold et al., *Trends Microbiol.* 2:437-443, 1994), suggesting that its presence in a recombinant adenoviral vector may be beneficial. Other adenovirus modifications have shown promise in delivering transgenes to target cells, with persistent transgene expression having resulted

therefrom (*see, e.g.* WO98/46781, WO98/46780, and WO98/46779 and Scaria et al., *J. Virol.*, 72:7302-7309, 1998). The lack of persistence in the expression of adenoviral vector-delivered transgenes may also be due to limitations imposed by the choice of promoter or transgene contained in the transcription unit (Guo et al., *Gene Therapy* 3:802-801, 1996; Tripathy et al., *Nature Med.* 2:545-550, 1996). Further optimization of minimal adenoviral vectors for persistent transgene expression in target cells and tissues also involves the design of expression control elements, such as promoters, which confer persistent expression to an operably linked transgene. Promoter elements, which function independently of particular viral genes to confer persistent expression of a transgene, allow the use of vectors containing reduced viral genomes.

In addition to containing the inverted terminal repeat sequences, PAV vectors also contain a *cis*-acting packaging sequence, normally located at the 5' end of the wild-type adenoviral genome. The packaging sequence contains seven functional elements, identified as A repeats (Schmid et al., *J. Virol.* 71:3375-3384, 1997).

Production of PAV or other minimal adenoviral vectors requires the provision of adenovirus proteins *in trans* which facilitate the replication and packaging of a PAV genome (and inserted foreign nucleic acid) into viral vector particles for use in gene transfer. Most commonly, such genes are provided by infecting the producer cell with a helper adenovirus containing the necessary genes. However, such viruses are potential sources of contamination of the PAV vector stock during purification if they are able to replicate and be packaged into viral particles. It is advantageous, therefore, to increase the purity of a PAV stock by reducing or eliminating any production of helper viruses which contaminate the preparation. Several strategies to reduce the production of helper viruses in the preparation of a PAV stock are disclosed in U.S. Patent No. 5,882,887 and international application No. PCT/US99/03483, filed February 17, 1999 both incorporated herein by reference. For example, the helper virus can contain mutations in the packaging sequence of its genome which prevent packaging, or may contain an oversized adenoviral genome which cannot be packaged.

Novel helper viruses which facilitate the production of pseudoadenoviral vectors (PAV) by providing essential viral proteins *in trans*, but which are packaging defective due to the inclusion of binding sequences for repressor proteins that prevent utilization of the packaging signals in the helper virus genome have been disclosed in
5 PC/US99/03483, filed February 17, 1999, incorporated herein by reference. The PCT application also provides PAV producer cell lines expressing such repressor proteins and to methods for the production of PAV using such helper viruses and producer cell lines.

Recently, PAV helper viruses have been described in which packaging of the
10 helper is reduced through the use of the *cre/lox* system (Parks et al., *Proc. Natl. Acad. Sci. USA* 93:13565-13570, 1996). Lox sites are placed at positions flanking the Ad packaging sequences in the helper viral genome, which is produced in conventional 293 cells. For PAV production, a *cre*-expressing 293 cell is employed. The helper genome can replicate and express viral genes so that the PAV genome can be
15 packaged, but the packaging sequences are deleted from the helper through the action of the cre protein.

However, methods of producing PAV have not been maximized; measurable amounts of helper virus can remain in vector preparations. In addition, current methods of PAV production are not readily scalable for larger scale uses.

20 The present invention provides an alternative adenoviral vector system with a novel large transgene packaging capacity in which the adenoviral genes deleted from the vector are expressed only in a novel producer cell. The producer cell used to manufacture the Ad vector and an advanced producer cell system for use in producing PAV, both in scalable amounts, are also provided.

25 Summary of the Invention

The present invention provides a partially-deleted adenoviral (termed "DeAd") vector in which the majority of adenoviral early genes required for virus replication are deleted from the vector and placed within a producer cell chromosome under the control of a conditional promoter. The deletable adenoviral genes that are placed in

the producer cell include E1A/E1B, E2, E4 (only ORF6 and ORF6/7 need be placed into the cell), pIX and pIVa2. E3 may also be deleted from the vector, but since it is not required for vector production, it can be omitted from the producer cell. The adenoviral late genes, normally under the control of the major late promoter (MLP),
5 are present in the vector, but the MLP has been replaced by a conditional promoter.

Conditional promoters suitable for use in DeAd vectors and producer cell lines include those with the following characteristics: low basal expression in the uninduced state, such that cytotoxic or cytostatic adenovirus genes are not expressed at levels harmful to the cell; and high level expression in the induced state, such that
10 sufficient amounts of viral proteins are produced to support vector replication and assembly. Preferred conditional promoters suitable for use in DeAd vectors and producer cell lines include the dimerizer gene control system, based on the immunosuppressive agents FK506 and rapamycin, the ecdysone gene control system and the tetracycline gene control system.

15 The invention also provides for producer cells suitable for commercial production of vectors, in which the adenoviral early genes deleted from the DeAd vector have been inserted into the cell's chromosome under the control of conditional promoters, such as the dimerizer system, based on the immunosuppressive agents FK506 and rapamycin, the ecdysone inducible system or the tetracycline gene control
20 system.

In a further aspect, the present invention also provides for improved PAV-producer cells derived from DeAd producer cells.

The invention also provides methods for making and using the vectors and producer cells of the invention.

25 Brief Description of the Drawings

Fig. 1 is a schematic representation of a DeAd vector genome.

Fig. 2 is a schematic representation of a DeAd vector producer cell utilizing the dimerizer system.

Fig. 3 is a schematic representation of a DeAd vector producer cell utilizing the ecdysone system.

Fig. 4 is a schematic representation of a DeAd vector producer cell utilizing the Tet-off system.

5 Fig. 5 is a schematic representation of a DeAd vector producer cell utilizing the Tet-on system.

Fig. 6 is a schematic representation of a PAV producer cell utilizing the dimerizer system.

10 Fig. 7 is a schematic representation of a PAV producer cell utilizing the ecdysone system.

Fig. 8 is a schematic representation of a PAV producer cell utilizing the Tet-off system.

Fig. 9 is a schematic representation of a PAV producer cell utilizing the Tet-on system.

15 Detailed Description of the Invention

The invention provides for a partially-deleted adenoviral (DeAd) vector in which a majority of the adenovirus early genes (E1-E4) required for virus replication have been deleted from the vector. The deleted genes, with the possible exception of E3, which is not required for replication, are inserted into a producer cell chromosome
20 under the control of a conditional promoter in order to facilitate vector production.

Conditional promoters suitable for use in DeAd vectors and producer cell lines include those with the following characteristics: low basal expression in the uninduced state, such that cytotoxic or cytostatic adenovirus genes are not expressed at levels harmful to the cell; and high level expression in the induced state, such that
25 sufficient amounts of viral proteins are produced to support vector replication and assembly.

DeAd vectors contain the adenovirus late genes, whose expression is normally under the control of the major late promoter (MLP). The MLP can be replaced by a

conditional promoter, including, *inter alia*, the dimerizer gene control system, the ecdysone gene control system and the tetracycline gene control system.

Partially-Deleted Adenoviral (DeAd) Vector

The key features of the DeAd vector system are: (1) the majority of adenovirus
5 early genes required for virus replication are deleted from the vector genome and placed within the producer cell chromosome under conditional promoter control, (2) adenovirus late genes, normally under the control of the major late promoter (MLP) are present in the vector, but are under the control of a conditional promoter which replaces the MLP, and (3) the producer cell is based on an established cell line chosen
10 for its capability to be grown at a commercial scale. Alternately, the MLP activity may be reduced by deleting the adenovirus IVa2 gene. The product of the IVa2 gene is a transcriptional activator of the MLP (Tribouley et al., *J. Virol* 68: 4450-4457, 1994; Lutz et al., *J. Virol* 70: 1396-1905, 1996). Through these vector genome alterations, two different objectives are accomplished. First, additional space is
15 created within the vector genome, such that inserts of approximately 12-15 kb of foreign nucleic acid (transgene) can be accommodated. This is approximately one third to one half the size of the transgene carrying capacity of PAV (up to 36 kb of foreign nucleic acid), but is large enough to carry a variety of complex genes. Second, expression of viral genes from the vector is eliminated, thus reducing the
20 possibility for immune or cytotoxic responses that may be harmful to the transfected cell.

The adenoviral genes that are deleted and placed within the producer cell genome include E1A/E1B, E2, E4 (only ORF6 and ORF6/7 placed in cell), pIX and pIVa2. The E3 genes are deleted from the vector genome but not included in the
25 producer cell since the gene products of E3 are not required for vector growth. The vector and producer cell are constructed in parallel, such that there are no overlapping sequences that permit restoration of viral genes to the vector genome through recombination. The adenoviral major late promoter (MLP) controls expression of all of the virion structural proteins encoded by the top strand of the vector genome. In

the present vectors, the MLP promoter is replaced with a conditionally expressed promoter by deleting all adenoviral sequences between position 358 of the adenovirus genome and the TATAA box of the MLP (Park and Shenk, *J. Virol.* 71:9600-9607, 1997), for example, nucleotides 358-6038 (*see* Example 1). In other embodiments of the invention, the deletion encompasses (1) nucleotides 358-6007 which deletes the MAZ site upstream from the TATAA box; (2) nucleotides 358-6012 which deletes the MAZ site upstream from the TATAA box and the TATAA box; or (3) nucleotides 358-6026 which deletes the MAZ site upstream from the TATAA box, the TATAA box and the adjacent MAZ/SP1 site. The numbering and sequence of the adenovirus, serotype 2 ("Ad2") genome, which is used to exemplify the present invention, is available from GenBank. The structure of a DeAd vector according to the invention is shown in Fig 1. In this vector there are no sequences to the left of the normal position of the MLP that would allow recombination with viral genes placed within the producer cell genome, and there are no sequences to the right of the normal position of the E2A promoter that would allow recombination with viral genes placed within the producer cell genome. While the present invention is exemplified with adenovirus of serotype 2 (Ad 2), other adenovirus serotypes can also be used to construct DeAd vectors, including, *inter alia*, Ad 5, Ad 6, Ad 9, Ad 12, Ad 15, Ad 17, Ad 19, Ad 20, Ad 22, Ad 26, Ad 27, Ad 28, Ad 30 and Ad 39. Ad 2, Ad 5, Ad 6 and Ad 17 are preferred.

The choice of the cell used to construct the producer cell is a key component of the DeAd vector system. The cell that is most commonly used to produce Ad vectors is the human 293 cell. This cell line was established by transfecting human embryonic kidney cells with fragments of the Ad5 genome and selecting for a transformed phenotype (Graham, F.L. et al., *J. Gen. Virol.* 36:59-72, 1997). This cell line contains approximately 3500 base pairs from the left end of Ad5 and expresses constitutively the E1 gene products, and thus supports replication of E1 deleted Ad vectors. However, there are limitations of the 293 cell. It was not selected for its adaptability to large-scale growth, that is required for commercial scale production of Ad vectors. In addition, the incorporation of additional viral genes into the 293 cell

genome has generally resulted in modified progeny cell lines from which the vector yield is reduced. Moreover, to date, the incorporation of the number of viral genes into the producer cell genome required for the DeAd vector system has not been reported. Nonetheless, there is a large body of experience on the production of
5 adenoviral vectors in 293 cells, including clinically used adenoviral vectors. Based on these criteria and the ability of 293 cells to support adenovirus replication, the cell lines used in the DeAd vector system include the 293 cell, the A549 cell line, and the KB cell line (all of which are available from ATCC).

Gene control elements

10 Conditional promoter systems used to control adenoviral genes, whether the genes are present within the vector genome or the producer cell genome, include *inter alia* the dimerizer gene control system based on the immunosuppressive agents FK506 or rapamycin, the ecdysone gene control system and the tetracycline gene control system. Other conditional promoter systems, including inducible promoters,
15 may also be used and are within the scope of the present invention. The adenovirus genes are linked to selectable drug resistance marker genes, introduced into cells through the use of standard transfection techniques, followed by selection with the appropriate drug selection scheme.

Dimerizer Gene Control System. The dimerizer gene control system (Rivera
20 et al., *Nature Med.* 2:1028-1032, 1996; Amara et al., *Proc. Natl. Acad. Sci. USA* 94: 10618-10623, 1997; both incorporated herein by reference) is based on two immunosuppressive agents, FK506 and rapamycin (Fischer et al., *Nature* 337:476-478, 1989; Harding et al., *Nature* 341:758-760, 1989; Siekierka, *Nature* 341:755-757, 1989; all incorporated herein by reference). Each of these agents binds tightly to the
25 cell protein FKBP12, a 12kd protein which can be expressed as a genetic fusion with other proteins and still maintain FK506 binding capability. The rapamycin/FKBP12 complex binds tightly to another cell protein called FRAP. Importantly, the binding capacity of FRAP for the rapamycin/FKBP12 complex is contained within a small domain of FRAP, called FRB, and this domain can be expressed as a genetic fusion

with other proteins and still retain its binding property for the rapamycin/FKBP12 complex.

Control of gene expression with this system requires (1) a minimal promoter (inactive on its own) placed downstream from specific control elements, (2) a gene
5 construct expressing from a constitutive promoter comprising a DNA binding domain (devoid of transcriptional activation activity) of a transcription factor that recognizes the aforementioned control elements, and (3) a gene construct expressing from a constitutive promoter comprising a transcriptional activation domain of a transcription factor devoid of DNA binding activity. The DNA binding domain and the
10 transcription activation domain proteins are expressed as genetic fusions with either FKBP12 or FRB. At least one FKBP12 or FRB domain, but frequently 2 to 4 domains, are included in the fusion constructs. In the absence of the dimerizer drug (FK506 or rapamycin), the two portions of the transcription factor are expressed within the cell but do not associate and, therefore, do not direct transcription.
15 Addition of the appropriate dimerizer causes the two portions of the transcription factor to associate with each other with high affinity and to induce transcription of genes operably linked to the minimal promoter and specific control elements described above in (1). If the separate DNA binding domains and activation domains are fused to FKBP12, then activation of transcription is accomplished through the use
20 of a dimer of FK506 called FK1012. If either the separate DNA binding domain or the activation domain is fused to FKBP12 and the other fused to FRB, then activation of transcription is accomplished through the use of rapamycin.

The DNA binding domain of choice is called ZFHD1, and was constructed from zinc fingers 1 and 2 from the Zif268 transcription factor linked to the
25 homeodomain from the Oct-1 protein (Pomerantz et al., *Science* 267: 93-96, 1995, incorporated herein by reference). The promoter controlled by this factor that is used in the DeAd vector system contains 12 repeats of the binding sequence for ZFDH1 placed adjacent to a minimal promoter. Examples of the minimal promoter include, the minimal CMV promoter, the minimal IL-2 promoter, or the minimal drosophila
30 hsp70 promoter. The producer cell is stably transfected with gene constructs

expressing the separate DNA binding domain and activation domain fusion gene constructs, as well as gene constructs in which the desired viral proteins are placed under control of the 12 repeat ZFDH1 promoter. The major late promoter (MLP) is replaced by the 12 repeat ZFDH1 promoter, as well. Transcription of adenoviral genes placed within the producer cell genome and of the viral structural proteins normally controlled via the major late promoter (MLP) is induced by the addition of either FK1012 or rapamycin or other related dimerizer agent.

Ecdysone Gene Control System. The control of gene expression in the DeAd vector system using the ecdysone regulated promoter system is carried out in an analogous manner. No et al., *Proc. Natl. Acad. Sci. USA* 93:3346-3351, 1996, incorporated here in by reference, have designed a system wherein gene expression from a minimal promoter is under strict control of separate steroid binding receptors that associate and activate transcription only in the presence of the insect hormone ecdysterone (or derivatives thereof). The DNA binding elements that are used are the ecdysone response elements (EcREs) and between 4 and 5 repeats of this element are used to control a minimal promoter with very low basal activity, such as the drosophila hsp70 promoter deleted of its own enhancer elements. Increased expression activity is provided by the insertion of consensus Sp1 binding elements between the EcREs and the minimal promoter. The precise arrangement of these sequence elements is defined in No et al.; *supra*. Expression from this promoter is controlled by the co-expression in the cell of two steroid receptor elements the mammalian retinoid X receptor (RXR) and a modified ecdysone receptor of insect origin (either VpEcR or VgEcR). Each of these receptors has the native transactivation domain replaced by the transactivation domain of a herpes virus protein, VP16. VgEcR is further altered such that it recognizes a modified response element (E/GRE) that is a hybrid between the glucocorticoid receptor (GR) and the ecdysone receptor (EcR). The structure of the E/GRE element is described in No et al., *supra* VpEcR participates with RXR in activation of EcRE-controlled genes, while VgEcR participates, along with RXR, in activation of E/GRE-controlled genes. Activation of gene expression occurs when the hormone ecdysone or analogs thereof,

such as muristerone A are administered to cells containing a complete set of the receptor gene constructs and the promoter-controlled gene or genes activated by the receptors.

Tetracycline Gene Control System. The control of gene expression in the
5 DeAd vector system using the tetracycline gene control system is carried out in an analogous manner. Gossen and Bujard, *Proc. Natl. Acad. Sci. USA* 89:5547-5551, 1992; and Gossen et al., *Science* 268:1766-1769, 1995, both incorporated here by reference, have designed a system wherein gene expression from a minimal promoter is under strict control of the tetracycline repressor (TetR), expressed as a fusion
10 protein with the herpes virus VP16 transcriptional activation domain (TetR/VP16). Two versions of the TetR/VP16 protein exist: the wild type TetR is active only in the *absence* of tetracycline or doxycycline, while a mutated form of TetR (reverse TetR or rTetR) is active only in the *presence* of doxycycline. When linked to VP16, the TetR form activates transcription only in the absence of tetracycline and the rTetR form
15 activates transcription only in the presence of doxycycline. Each of these transcriptional control factors, TetR/VP16 and rTetR/vp16 controls the expression of genes linked to a minimal promoter cloned adjacent to tetracycline transcriptional regulatory elements (TRE).

Alternatively gene expression can be controlled using a chimeric transactivator
20 (e.g. GL_p65) which consists of a mutated progesterone receptor-ligand binding domain fused to a GAL4 DNA binding domain and part of the activation portion of the human p65 protein (a component of the NF- κ B complex). In the presence of mefepristone (RU-486), an antiprogesterin, the chimeric transactivator binds to target nucleic acid containing a 17 mer GAL4 binding site inserted therein, thereby resulting
25 in inducible expression. (Burcin et al., *Proc. Natl. Acad. Sci. USA* 96: 355-360, 1999; Wang et al., *Gene Ther.* 4:432-441, 1997; Robbins et al., *Pharmacol. Ther.* 80: 35-47, 1998 all incorporated herein by reference).

The present invention also includes methods for providing a transgene to the cells of an individual and having the transgene expressed therein to produce a
30 phenotypic alteration correlated with the transgene product comprising introducing

into the cells a DeAd vector comprising a transgene operably linked to expression control sequences, wherein the DeAd vector is taken up by target cells, the transgene is delivered to and expressed therein to produce the phenotypic alteration correlated to the transgene product.

- 5 Polynucleotides/transgenes are inserted into DeAd vectors of the invention using methods well known in the art. Transgenes are defined herein as nucleic acid molecules or structural genes that encode a particular polypeptide or protein or a ribozyme or an antisense RNA or the like. Transgenes encoding polypeptides or proteins include, *inter alia*, those coding for enzymes, *e.g.* human lysosomal enzymes,
- 10 such as α -galactosidase A and β -glucocerebrosidase, hormones, growth factors, cytokines, antigens, and such specific proteins such as CFTR, alpha1-antitrypsin, soluble CD4, adenosine deaminase, Herpes Simplex Virus thymidine kinase, the tumor antigens gp100, MART-1 and TRP-2; and clotting factors, such as factor VIII, factor IX, factor VII and Von Willebrand factor. Representative human lysosomal
- 15 enzymes in accordance with the present invention are provided in Table I. References relating to isolation and characterization of the lysosomal enzymes and nucleic acid molecules (transgenes) encoding said enzymes in Table I may be found in Scriver et al., *The Metabolic Basis of Inherited Disease*, 7th Ed., vol. II, pp. 2427-2879, McGraw Hill, 1995, incorporated herein by reference.

20 Table I. Lysosomal storage diseases and associated enzymatic defects

Disease	Enzymatic Defect
Pompe disease	acid α -glucosidase (acid maltase)
MPSI* (Hurler disease)	α -L-iduronidase
MPSII (Hunter disease)	iduronate sulfatase
25 MPSIII (Sanfilippo)	heparan N-sulfatase
MPS IV (Morquio A)	galactose-6-sulfatase
MPS IV (Morquio B)	acid β -galactosidase
MPS VII (Sly disease)	β -glucuronidase
I-cell disease	N-acetylglucosamine-1-phosphotransferase

	Schindler disease	α -N-acetylgalactosaminidase (α -galactosidase B)
	Wolman disease	acid lipase
	Cholestrol ester storage disease	acid lipase
	Farber disease	lysosomal acid ceramidase
5	Niemann-Pick disease	acid sphingomyelinase
	Gaucher disease	β -glucosidase (glucocerebrosidase)
	Krabbe disease	galactosylceramidase
	Fabry disease	α -galactosidase A
	GM1 gangliosidosis	acid β -galactosidase
10	Galactosialidosis	β -galactosidase and neuraminidase
	Tay-Sach's disease	hexosaminidase A
	Sandhoff disease	hexosaminidase A and B

*MPS = mucopolysaccharidosis

By way of example, in order to insert the transgene into the vector, the

15 transgene and vector nucleic can be contacted, under suitable conditions, with a restriction enzyme to create complementary ends on each molecule that can pair with each other and be joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of the restricted polynucleotide. These synthetic linkers contain nucleic acid sequences that correspond to a particular restriction site in

20 the vector nucleic acid. Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV

25 for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are well known and available in the art.

As used herein, "expression" refers to the process by which polynucleotides/transgenes are transcribed into mRNA and then translated into peptides, polypeptides, or proteins. If the transgene encodes a ribozyme or an antisense RNA, expression refers to the synthesis of the ribozyme or antisense molecule. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eukaryotic host is selected. Regulatory elements required for expression include promoter sequences that bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the *lac* promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al., *Molecular Cloning, A Laboratory Manual 2d Ed.* (Cold Spring Harbor, NY, 1989), or Ausubel et al., *Current Protocols in Molecular Biology* (Greene Assoc., Wiley Interscience, NY, NY, 1995). Similarly, a eukaryotic expression vector, be it a virus or a plasmid, includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the protein encoded by the polynucleotide/transgene.

Preparations of the transgene encoding a human lysosomal enzyme, *e.g.*, α -galactosidase A, can be incorporated into the DeAd vectors of the invention for delivery into an individual's cells, *e.g.*, a Fabry individual, using methods that are known in the art. See, for example, Finkel and Epstein, *FASEB J.* 9:843-851, 1995; Feldman and Steg, *Cardiovascular Res.* 32:194-207, 1996.

Alternatively, the DeAd vectors comprising a transgene encoding α -galactosidase A may be delivered to Fabry knockout mice to provide biologically active enzyme thereto and reduce the levels of stored lipid -- glycosphingolipid globotriasylceramide (GL3) -- which accumulates in the absence of active enzyme. Recently, a Fabry knockout transgenic mouse demonstrating a deficiency in this

enzyme activity has been made (Ohshima et al., *Proc. Natl. Acad. Sci., USA* 94:2540-2544, 1997). Such knockout mice display a complete lack of α -galactosidase A activity and accumulate GL3 in their tissues. Lipid analysis of the liver and kidneys of the knockout mice revealed a marked accumulation of GL3 over time, indicating
5 the similarity of the pathophysiological process in the mutant mice and in patients with Fabry disease. *Id.* Thus, the Fabry knockout mice provide an excellent model for the human disease.

In the DeAd vectors of the present invention, the transgene is operably linked to expression control sequences, *e.g.*, a promoter that directs expression of the
10 transgene. As used herein, the phrase "operatively linked" refers to the functional relationship of a polynucleotide/transgene with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of a nucleic acid to a promoter refers to the physical and functional relationship between the polynucleotide
15 and the promoter, such that transcription of DNA is initiated from the promoter by an RNA polymerase that specifically recognizes and binds to the promoter, and wherein the promoter directs the transcription of RNA from the polynucleotide.

Promoter regions include specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. Additionally, promoter
20 regions include sequences that modulate the recognition, binding and transcription initiation activity of RNA polymerase. Such sequences may be *cis* acting or may be responsive to *trans* acting factors. Depending upon the nature of the regulation, promoters may be constitutive or regulated. Examples of promoters are SP6, T4, T7, SV40 early promoter, cytomegalovirus (CMV) promoter, mouse mammary tumor
25 virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, phosphoglycerate kinase (PGK) promoter, and the like. Alternatively, the promoter may be an endogenous adenovirus promoter, for example the E1a promoter or the Ad2 major late promoter (MLP). Similarly, those of ordinary skill in the art can construct adenoviral vectors utilizing endogenous or heterologous poly A addition
30 signals.

The DeAd vectors of the invention may be targeted to specific cells by linking a targeting molecule to the vector, *e.g.* as disclosed in international application No. PCT/US99/02680 filed February 8, 1999, incorporated herein by reference. A targeting molecule is any agent that is specific for a cell or tissue type of interest, including for example, a ligand, antibody, sugar, receptor, or other binding molecule. The ability of targeted vectors renders invention vectors particularly useful in the treatment of lysosomal storage disorders. For example, including a targeting molecule, such as VEGF or an antibody to a VEGF receptor can provide targeting to vascular endothelial cells in individuals with Fabry's disease.

10 In addition, the DeAd vectors can be complexed with a cationic amphiphile, such as a cationic lipid, polyL-lysine (PLL), and diethylaminoethyl-dextran (DEAE-dextran) to provide increased efficiency of viral infection of target cells (*See, e.g.*, WO98/22144, incorporated herein by reference). Representative cationic lipids include those disclosed, for example, in U.S. Patent Nos. 5,238,185 and 5,767,099, 15 with preferred lipids being GL-67 (N⁴-spermine cholesteryl carbamate), GL-53 (N⁴-spermine cholesteryl carbamate), and GL-89 (1-(N⁴-spermine)-2,3-dilaurylglycerol carbamate).

DeAd vectors complexed with DEAE dextran are particularly preferred. In addition, since repeat administration of a viral vector can result in an immune response to the vector, thereby limiting its effectiveness in delivering the gene to affected cells, adenovirus and other viral vectors may be polymer-modified, *e.g.*, 20 complexed with polyethylene glycol (PEG), to reduce viral immunogenicity and allow for repeat administration of the vector (*See, e.g.*, WO/98/44143, incorporated herein by reference). Also DeAd vectors complexed with a cationic molecule, preferably 25 DEAE, and a polyalkylene glycol polymer, *e.g.* PEG, as disclosed in U.S. provisional patent application Serial NO. 60/097,653, filed August 24, 1998, are also contemplated herein. Alternatively, the vector may be administered with an immunosuppressive agent to reduce the immune response to repeated vector administration. In addition, combinations of the above approaches may be used.

Transfer of the transgene to the target cells by the DeAd vectors of the invention can be evaluated by measuring the level of the transgene product in the target cell and correlating a phenotypic alteration associated with transgene expression. For example, expression of a CFTR transgene in target cells from an individual with
5 cystic fibrosis is correlated with production of a functional chloride ion channel in such cells that may be measured by techniques known in the art. The level of transgene product in the target cell directly correlates with the efficiency of transfer of the transgene by DeAd vectors. Any method known in the art can be used to measure transgene product levels, such as ELISA, radioimmunoassay, assays using an
10 fluorescent and chemiluminescent enzyme substrates.

Expression of the transgene can be monitored by a variety of methods known in the art including, *inter alia*, immunological, histochemical and activity assays. Immunological procedures useful for *in vitro* detection of the transgene product in a sample include immunoassays that employ a detectable antibody. Such
15 immunoassays include, for example, ELISA, Pandex microfluorimetric assay, agglutination assays, flow cytometry, serum diagnostic assays and immunohistochemical staining procedures which are well known in the art. An antibody can be made detectable by various means well known in the art. For example, a detectable marker can be directly or indirectly attached to the antibody.
20 Useful markers include, for example, radionuclides, enzymes, fluorogens, chromogens and chemiluminescent labels.

For *in vivo* imaging methods, a detectable antibody can be administered to a subject and the binding of the antibody to the transgene product can be detected by imaging techniques well known in the art. Suitable imaging agents are known and
25 include, for example, gamma-emitting radionuclides such as ^{111}In , $^{99\text{m}}\text{Tc}$, ^{51}Cr and the like, as well as paramagnetic metal ions, which are described in U.S. Patent No. 4,647,447. The radionuclides permit the imaging of tissues by gamma scintillation photometry, positron emission tomography, single photon emission computed tomography and gamma camera whole body imaging, while paramagnetic metal ions
30 permit visualization by magnetic resonance imaging.

The DeAd vectors of the present invention can be assayed for the ability to provide persistence of transgene expression *in vivo* using the DeAd vectors in a recognized animal model system. Such a model may be chosen with reference to such parameters as ease of delivery, identity of transgene, relevant molecular assays and
5 assessment of clinical status. Where the transgene encodes a protein whose lack is associated with a particular disease state, an animal model which is representative of the disease state may optimally be used in order to assess a specific phenotypic result correlated with the presence of biologically active transgene product *e.g.*, Fabry
knockout mice (as disclosed in international No. PCT/US98/22886, filed October 29,
10 1998), may be used to assay the ability of DeAd vectors comprising an α -galactosidase A transgene to reduce the levels of GL-3 in such mice.

Relevant animals in which the transgene expression system may be assayed include, but are not limited to, mice, rats, monkeys and rabbits. Suitable mouse strains in which the transgene expression system may be tested include, but are not
15 limited to, C3H, C57B1/6 (wild-type and nude) and Balb/c (available from Taconic Farms, Germantown, New York).

Where it is desirable to assess the host immune response to DeAd vector administration, testing in immune-competent and immune-deficient animals may be compared in order to define specific adverse responses generated by the immune
20 system. The use of immune-deficient animals, *e.g.*, nude mice, may be used to characterize vector performance and persistence of transgene expression, independent of an acquired host response and to identify other determinants of transgene persistence.

In order to determine the persistence of DeAd vectors in the host, one skilled
25 in the art can assay for the presence of these vectors by any means which identifies the transgene (and its expression), for example, by assaying for transgene or nucleic acid encoding the immunomodulatory molecule mRNA level by RT-PCR, Northern blot or S1 analysis, or by assaying for transgene protein expression by Western blot, immunoprecipitation, or radioimmunoassay. Alternatively, the presence of the DeAd
30 vector or the desired transgene DNA sequences *per se* in a host can be determined by

any technique that identifies DNA sequences, including Southern blot or slot blot analysis, or other methods known to those skilled in the art. Where the vector contains a marker gene, *e.g.*, *lacZ* coding for *E. coli*, β -galactosidase, the presence of the vector may be determined by these same assays or a specific functional assay that
5 screens for the marker protein (*e.g.*, X-gal). The persistence of a vector of the invention in the host can also be determined from the continued observation of a phenotypic alteration conferred by the administration of the DeAd vector containing the transgene, *e.g.*, the improvement or stabilization of pulmonary function following administration of a vector containing the CFTR gene to an individual with cystic
10 fibrosis or the reduction in stored GL3 lipid in an individual with Fabry's disease. Spirometry can be used for pulmonary function tests (PFT) in CF individuals. Demonstration of the restoration of chloride ion channel function in the DeAd vector-treated cells of a CF patient can also be used to assess the persistence of the transgene CFTR (Zabner et al., J. Clin. Invest. 97:1504-1511, 1996).

15 The present invention also encompasses compositions containing the DeAd vectors of the invention which can be administered in an amount effective to deliver a desired transgene and/or nucleic acid to target cells, achieve transgene expression therein and obtain a phenotypic alteration correlated with the transgene product. The compositions can include physiologically acceptable carriers, including any relevant
20 solvents. As used herein, "physiologically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. Except insofar as any conventional media or agent is incompatible with the active ingredient, *i.e.*, the adenoviral vectors of the invention, its use in the compositions of the invention is contemplated.

25 Routes of administration for the compositions containing the DeAd vectors include conventional and physiologically acceptable routes such as direct delivery to target cells, organs or tissues, intranasal, intravenous, intramuscular, subcutaneous, intradermal, oral and other parenteral routes of administration. The vectors may also be administered via inhalation of liquid or dry powder aerosols (*e.g.* as disclosed in

U.S. provisional patent application Serial No. 60/110,899, filed December 4, 1998, incorporated herein by reference.

The invention is further directed to methods for using the compositions of the invention *in vivo* or *ex vivo* applications in which it is desirable to deliver one or more
5 transgenes into cells using the DeAd vectors of the invention so as to provide persistent expression of a transgene encoding a biologically active molecule therein. *In vivo* applications involve the direct administration of an adenoviral vector of the invention formulated into a composition to the cells of an individual. *Ex vivo* applications involve the transfer of the adenoviral vector directly to harvested
10 autologous cells which are maintained *in vitro*, followed by readministration of the transduced cells to a recipient.

Example 1 - DeAd Vector

The invention is explained more fully in regard to construction of a DeAd vector from an adenovirus of serotype 2 (Ad2). The Ad 2 DeAd genome is modified
15 using conventional molecular cloning methods (See, e.g. Ausubel, F.M. et al., eds., 1987-1996, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. New York; Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), *DNA Cloning: A Practical Approach*, MRL Press, Ltd., Oxford, U.K. Vol. I, II, 1985,
20 incorporated herein by reference) to achieve a vector with the characteristics as shown in Fig 1. Nucleotides between 358 and 6038 (numbering and sequence of Ad2 available from GenBank) of the adenovirus genome are deleted to remove the E1 coding region, the pIX gene, the pIVa2 gene, and the MLP promoter. The E2A coding region is deleted by removal of nucleotides 22666 to 23960; this removes the
25 first ATG of the E2A protein encoding sequence, as well as a large portion of the protein coding region, without affecting genes encoded by the opposite strand of the virus. The E3 region is deleted by removal of nucleotides 27971 to 30937; this removes all E3 coding regions. The E4 region is deleted by removal of nucleotides 32815 to 35977; this removes all E4 coding regions. Through these deletions, the

transgene packaging size of the vector is increased to approximately 12kb. The DeAd vector genome is further modified by positioning the dimerizer controlled promoter, the ecdysone controlled promoter, or the tetracycline (doxycycline) controlled promoter in place of the MLP, *i.e.*, just upstream from position 6038.

5 Example 2 - Conditional expression of Ad late genes; deletion of VA RNA genes.

Cells infected with adenoviruses synthesize large amounts of two low molecular weight RNAs, designated VAI and VAII, during the late phase of infection. The genes coding for these two RNAs are located between 29.3 and 30.8 map units on the adenovirus chromosome (Furtado et al., *J. Virol.* 63: 3423-3434, 1989,
10 incorporated herein by reference). The VA RNA genes are transcribed by RNA polymerase III and contain intragenic transcription control regions. The VAI RNAs are produced in much larger amounts at late times after infection compared to VAII RNA (40:1 ratio). Adenovirus mutants that do not produce VAI RNA grow poorly because VAI RNA is required for efficient translation of viral mRNAs at late times
15 after infection (Thimappaya et al., *Cell* 31:543-551, 1982, incorporated herein by reference). During adenovirus infection, the VA RNAs block activation of the double-stranded RNA (dsRNA)-activated kinase, also called eIF-2 α kinase (Kitajewski, et al., *Cell* 45:195-200; O'Malley et al., *Cell* 44:391-400, 1986, both
20 incorporated herein by reference). In the absence of VA RNAs, the (dsRNA)-activated kinase gets activated and phosphorylates the protein synthesis initiation factor eIF-2, thus inhibiting protein synthesis at late times in infected cells.

Deletions are made in the DeAd vector genome between 29.3 and 30.8 map units on the adenovirus genome, corresponding to Ad2 nucleotides 10600 to 11030, in order to delete the VA RNAI and VA RNAII genes. The resulting DeAd vectors can
25 be grown to high titers in 293 cells that have been engineered to express VA RNAs. DeAd vectors, in which the VA RNAs have been deleted, exhibit decreased synthesis of late proteins *in vivo*, leading to a decreased cellular immune response *in vivo* and better vector persistence *in vivo*. Such a VA RNA deleted vector is useful as a helper vector for construction of PAV (completely deleted Ad vectors), because the small

amounts of helper vector contamination that usually exists in PAV preparations are VA RNA deleted.

In the case where the major late promoter has been silenced in the DeAd vector, or has been completely deleted (PAV), inclusion of the VA RNA genes in the
5 vector improves transgene expression.

Example 3 - DeAd Vector Producer Cells

A DeAd vector producer cell (Fig. 2) using the dimerizer gene control system is generated such that it has stably associated in its genome: (1) a gene encoding a transcription activation domain (the activation domain of the NFkB p65 subunit is preferred) expressed as a fusion protein with multiple copies of either the FKBP12
10 binding domain or the FRB binding domain (3 copies of either are preferred), (2) a gene encoding the ZFHD1 DNA binding domain (Pomerantz et al., *Science* 267:93-96, 1995, incorporated herein by reference) expressed as a fusion protein with multiple copies of the FKBP12 binding domain or of the FRB binding domain (3
15 copies of either are preferred), (3) the coding sequences for the E1A/E1B region and pIX (nucleotides 498-4061 of the adenovirus genome), the pIVa2 gene (nucleotides 4050-5708), E4 ORF6 and ORF6/7 (nucleotides 32196-34082), and the entire E2 region (nucleotides 23960-4000) with each of these genes being placed under transcriptional control of the controllable promoter system of Rivera et al., *Nature*
20 *Med.* 2:1028-1032, 1996 and Amara et al., *Proc. Natl. Acad. Sci. USA* 94: 10618-10623, 1997, incorporated herein by reference (Fig. 2).

A DeAd vector producer cell (Fig. 3) using the ecdysone gene control system is generated such that it has stably associated in its genome: (1) a gene encoding the RXR receptor (2) a gene encoding the VpEcR or VgEcR receptor, (3) the coding
25 sequences for the E1 region and pIX (nucleotides 498-4061), the pIVa2 gene (nucleotides 4050-5708), E4 ORF6 and ORF6/7 (nucleotides 32196-34082), and the entire E2 region (nucleotides 23960-4000) with each of these genes being placed under transcriptional control of the ecdysone gene control system of No et al., *Proc. Natl. Acad. Sci. USA* 93:3346-3351, 1996, incorporated herein by reference.

A DeAd vector producer cell using the tetracycline gene control system is generated such that it has stably associated in its genome: (1) a gene encoding the tetracycline repressor with the herpes virus VP16 transactivation domain (TetR/VP16), (2) the coding sequences for the E1A/E1B region and pIX (nucleotides 498-4061), the Iva2 gene (nucleotides 4050-5708), E4 ORF6 and ORF6/7 (nucleotides 32196-34082), and the entire E2 region (nucleotides 23960-4000), with each of these genes being placed under transcriptional control of the tetracycline regulatory element (TRE) linked to the minimal CMV promoter. This system is referred to as the Tet-off system wherein the TetR/VP16 factor is inactive in the presence of tetracycline (or doxycycline), thus expression of the controlled genes is prevented when tetracycline or doxycycline is present. Removal of tetracycline or doxycycline leads to induction of expression of the controlled genes. (Fig. 4)

The alternative tetracycline gene control system, the Tet-on system, is similar to the Tet-off system except that a mutated Tet repressor is used. This tet repressor, called the reverse Tet repressor (rTetR), is active in the presence of doxycycline. In this case a DeAd vector producer cell using the tetracycline gene control system is generated such that it has stably associated in its genome: (1) a gene encoding the tetracycline-controlled transactivator expressed as a fusion of the reverse tetracycline repressor with the herpes virus VP16 transactivation domain (rTetR/VP16), (2) the coding sequences for the E1A/E1B region and pIX (nucleotides 498-4061), the Iva2 gene (nucleotides 4050-5708), E4 ORF6 and ORF6/7 (nucleotides 32196-34082), and the entire E2 region (nucleotides 23960-4000), with each of these genes being placed under transcriptional control of a compound promoter (Gossen and Bujard, *Proc. Natl. Acad. Sci. USA* 89:5547-5551, 1992) consisting of the tetracycline regulatory element (TRE) linked to the minimal CMV promoter. With this system, the tetracycline-controlled genes are silent in the absence of tetracycline and their expression is turned on when doxycycline is present. (Fig. 5).

Example 4 - Construction of DeAd Vector Containing a Transgene

A functional DeAd vector containing a transgene suitable for transfer of the functioning gene (*e.g.* CFTR; α -galactosidase A) to a target cell for expression therein is constructed and produced by the following procedure. First the transgene encoding

5 a biologically active (*e.g.* CFTR) or enzymatically active (*e.g.* α -galactosidase A) or immunologically active protein or a reporter gene (*e.g.* β -galactosidase) operably linked to a promoter element, such as the CMV promoter or any other desired promoter suitable for use with the transgene to direct its expression (*e.g.* PGK, adenovirus E1A), is cloned into the region downstream of position 358 in the DeAd

10 vector backbone shown in Fig. 1 using conventional molecular cloning methods (Ausubel, F.M., et al., ed., 1987-1996, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. New York; Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, *DNA Cloning: A Practical Approach*,

15 MRL Press, Ltd., Oxford, U.K. Vol. I, II).

The DeAd vector genome DNA comprising the transgene is transfected into any of the producer cells of Example 2 using standard calcium phosphate precipitation or lipid mediated gene transfection and using between 0.5 and 10 ug of DNA. Transcription of the major late transcription unit within the vector (MLP replaced by

20 conditional promoter) and of the stably associated viral genes is induced by the addition of rapamycin (between 2nM and 10nM rapamycin) or FK1012 dimerizer (between 5uM and 50uM) approximately, or by the addition of ecdysone (1uM), depending on which gene control system is being employed (Fig. 2 or Fig. 3). These inducers cause the association of the separate gene control elements of Rivera et

25 al. (*Nature Med.* 2:1028-1032, 1996) or Amara et al. (*Proc. Natl. Acad. Sci. USA* 94: 10618-10623, 1997) or No et al. (*Proc. Natl. Acad. Sci. USA* 93:3346-3351, 1996), which association generates active transcription factors that direct the expression of Ad structural proteins and regulatory proteins, leading to the packaging of the recombinant DeAd vectors containing the transgene or reporter gene of interest.

In the case of producer cells utilizing the tetracycline gene control system, transcription of the major late transcription unit within the vector (MLP replaced by conditional promoter) and the stably associated viral genes is controlled by tetracycline or doxycycline levels, depending on whether the Tet-off or Tet-on system is used. When the Tet-off system is used, the presence of tetracycline in the medium at between 0.1 and 1 µg per ml prevents expression from tetracycline controlled promoters. Thus, for construction of a DeAd vector and its subsequent propagation, doxycycline is included in the cell culture medium (Gossen et al., *Science* 268:1766-1769, 1995). Use of the tetracycline gene control system in either of these ways leads to the expression of adenovirus structural and regulatory proteins, which leads to the packaging of the recombinant DeAd vectors containing the transgene or reporter gene of interest.

The viral vectors thus produced are purified by conventional methods used for adenovirus purification, such as CsCl centrifugation or column chromatography (WO97/08298; U.S. Patent No. 5,837,520). The vectors may be further propagated by infection of the producer cell and addition of the rapamycin or FK1012, ecdysone or tetracycline (doxycycline) to induce expression of the required Ad genes.

Example 5 - PAV Producer Cell

PAV vectors contain no viral protein coding sequences and therefore their growth is dependent on providing all of the viral functions *in trans* (See e.g. U.S. Patent No. 5,882,877, incorporated herein by reference). Currently these functions are provided by a combination of E1 sequences stably associated with the producer cell (e.g. 293 cells) and from helper virus genomes that are co-transfected or infected along with PAV (See, e.g., U.S. Patent No. 5,882,877 and international application No. PCT/US99/03483 filed February 17, 1999, both incorporated herein by reference). This process, while effective for research purposes and small scale production, is limited for large commercial scale production. Thus it is advantageous to generate a producer cell line that has the capacity to (1) package PAV genomes transfected into the cell as has been done during the original packaging of new PAVs

at small scale, (2) amplify PAV vectors for research and proof-of-principle experiments, and (3) allow large scale growth for rigorous pre-clinical characterization and for commercial production. The cell lines described in Example 2 above provide many of the viral functions that are required for virus growth, but
5 they lack the ability to provide the major structural virion proteins. These proteins are encoded within the DeAd vector genome and are expressed only upon induction with dimerizers, ecdysone or tetracycline (doxycycline).

New PAV producer cells capable of providing all required functions for packaging of PAV are obtained by introducing, in addition to the adenovirus genes
10 already present in the cell lines of Example 2, the major late transcription unit, placed under control of dimerizer gene control (Fig. 6) or ecdysone gene control (Fig. 7), or tetracycline gene control (Figs. 8 and 9). To accomplish this, a portion of the DeAd vector genome is used. The Ad2 DeAd vector genomic sequences from position 6040 to 32815, and bearing each of the deletions shown within this genomic segment (Fig.
15 1), are placed under control of the dimerizer, ecdysone or tetracycline gene control elements as described in Example 2. Thus the PAV producer cells have stably associated in their genomes the following adenovirus genes: the coding sequences for the E1 region and pIX (nucleotides 498-4061 of the adenovirus genome), the pIVa2 gene (nucleotides 4050-5708), E4 ORF6 and ORF6/7 (nucleotides 33178-34082), the
20 entire E2 region (nucleotides 23960-40000), and the structural virion genes encoded by the L1 through L5 transcripts from the top strand of the adenovirus genome, with each of these genes being placed under transcriptional control of the controllable promoter system of Rivera et al. (*Nature Med.* 2:1028-1032, 1996) and Amara et al. (*Proc. Natl. Acad. Sci. USA* 94: 10618-10623, 1997) or the inducible ecdysone system
25 of No et al. (*Proc. Natl. Acad. Sci. USA* 93:3346-3351, 1996), or the tetracycline control system of Gossen et al. (*Proc Natl. Acad. Sci. USA* 89:5547-5551, 1992; *Science* 268:1766-1769, 1995).

Example 6 - Construction of a DeAd Vector Containing a CFTR Transgene, a human α -galactosidase A Transgene, an EPO Transgene, a Factor IX Transgene, a Factor VIII Transgene, or lacZ Reporter Gene and Transfer of the Gene to Recipient Cells

A DeAd vector comprising the functional CFTR encoding transgene for
5 transferring a functional CFTR coding sequence (Riordan et al., *Science* 245:1066-1073, 1989; U.S. Patent No. 5,876,974) to cells of an individual with cystic fibrosis, nucleic acid encoding human α -galactosidase A (e.g. U.S. Patent No. 5,658,567; PCT/US98/22886, filed October 29, 1998) the nucleic acid encoding erythropoietin (EPO) (U.S. Patent 4,703,008), the factor IX coding sequence (U.S. Patent
10 4,994,371), or the factor VIII coding sequence with the B chain present or deleted (Toole et al., PNAS USA 83:5939, 1986), is constructed as provided in Example 4 by cloning the relevant nucleic acid (transgene encoding CFTR, α -galactosidase A EPO, factor IX or factor VIII) operably linked to a promoter, such as the CMV promoter, the PGK promoter or other promoter suitable for expression of the nucleic acid
15 (transgene), preferably the CMV promoter, into the region downstream of position 358 in the DeAd vector of Example 1 (Fig. 1), using conventional cloning techniques (Ausubel, F.M. et al., eds., 1987-1996, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. New York; Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring
20 Harbor, New York; Glover, D.M. (ed.), 1985, *DNA Cloning: A Practical Approach*, MRL Press, Ltd., Oxford, U.K. Vol. I, II; or as described in U.S. Patent No. 5,670,488, Armentano et al., *J. Virol.* 71: 2408-2416, 1997, Rich et al., *Hum. Gene Ther.* 4: 461-476, 1993, all of which are incorporated herein by reference.

DeAd vectors comprising the CFTR transgene are propagated in any of the
25 producer cell lines described in Example 3 (Figs. 2, 3 and 4), released from the cells by suitable techniques, such as cell lysis and purified by CsCl gradient centrifugation as described in Zabner et al., *Nature Genetics* 6:75-83, 1994, incorporated herein by reference. Gene transfer of the CFTR gene to recipient cells is assayed by exposing cells in culture, preferably airway epithelial cells to the recombinant DeAd
30 vectors/CFTR at a dose of between 1 to 1000 virions/cell. For in vivo administration,

the DeAd/CFTR vector is administered by aerosol or other topical administration method to airway epithelia cells of a suitable animal models (*e.g.* cotton rats, primates) or to individuals with cystic fibrosis (*see, e.g.* U.S. Patent No. 5,670,488 and Zabner et al., *J. Clin. Invest.* 97: 1504-1511, 1996, incorporated herein by reference).

Expression of the CFTR transgene in treated cells, animals and individuals is detected by measurement of vector specific CFTR mRNA transcript (*see, e.g.* Kaplan et al., *Hum. Gene Ther.* 8:45-56, 1997, incorporated herein by reference) or phenotypic alteration (monitored by the presence of a functional chloride ion channel produced by the functional CFTR) in recipient cells (U.S. Patent No. 5,670,488 and Zabner et al, 1996, *supra*, Jiang et al., *Hum. Gene Ther.* 8: 671-680, 1997, incorporated herein by reference).

Expression of the EPO, factor IX and factor VIII transgenes in host cells transfected with DeAd vectors comprising said transgenes may be detected by any means known to those of skill in the art, including detection of RNA transcripts and protein production. Phenotypic alterations correlating with expression of the relevant transgene may also be assessed. For example, expression of EPO is measured by increased RBC production in an individual; expression of factors IX and VIII are monitored by measuring clotting in the individual.

Similarly, a DeAd vector comprising a *lacZ* reporter gene encoding β -galactosidase is made. Effective gene transfer and expression of the *lacZ* gene from the DeAd/*lacZ* vector to target cells or tissues is detected using an X-gal assay as disclosed in (Armentano et al., *J. Virol.* 71:2408-2416, 1997; Rich et al., *Hum. Gene Ther.* 4: 461-476, 1993; U.S. Patent No. 5,670,488, incorporated herein by reference).

A DeAd/ α galA vector is constructed, as provided in Example 4, by inserting a transgene encoding human α -galactosidase A operably linked to the CMV promoter into the DeAd vector of Example 1. The adenovirus EIA or PGK promoter may also be used to drive the expression of the α -galactosidase A transgene. Fibroblasts from normal and Fabry individuals, infected with DeAd/ α galA vectors produce enzymatically active α -galactosidase A, which may be assayed in cell lysates and

spent culture medium using the fluorescent substrate 4-methylumbelliferyl- α -D-galactopyranoside (4-mu- α -gal).

Example 7: Tissue distribution of α -galactosidase A in Fabry knockout mice after administration of DeAd/ α -gal A

- 5 The DeAd/ α galA vector of Example 6 is injected into the tail vein of female Fabry knockout mice at a dose of from 5×10^7 IU to 5×10^{10} IU. Mice are sacrificed after 3, 14 or 28 days. An ELISA is used to detect levels of α -galactosidase A activity in various organs. Intravenous injections of virus results in high levels of α -galactosidase A in all organs tested (10-100 fold).
- 10 DeAd/ α galA is injected into the right quadriceps muscle group of female Fabry knockout mice at a dose of 9×10^7 to 9×10^{10} IU. The mice are sacrificed after 5, 15 or 25 days. An ELISA is used to detect levels of α -galactosidase A in various organs. Intramuscular injections of virus results in significant levels of enzyme at the site of injection, as well as moderate enzyme levels in other sites, such as liver and
- 15 spleen, indicating that infected cells at the injection site secrete enzyme that is taken up by cells in other tissues.

Significant levels of active enzyme persist for some time after administering the DeAd/ α galA vector, at least up to about a month.

Example 8: Short term time course showing reduction of GL3 levels in Fabry mice intravenously administered DeAd/ α galA.

- 20 Female Fabry mice between 3 and 8 months of age (n=12, for each group) are injected via the tail vein with a dose (between 5×10^7 and 1.7×10^{11} IU of DeAd/ α galA vector). The mice are sacrificed at 3, 7 or 14 days post injection (n=4 per time point per dose). Two naive female Fabry mice (3 months and 8 months of age)
- 25 are sacrificed on day 3 for reference for GL3 levels in untreated mice. A blood sample is collected at the time of sacrifice to measure α -galactosidase A activity. Upon sacrifice, the animals are perfused with PBS and various organs collected. The organs are divided into two parts, one to assay for α -galactosidase A activity via an

ELISA specific for human α -galactosidase A and the other extracted and assayed for GL3 using an ELISA-type assay specific for GL3. The data are normalized to the weight of the tissue sample.

- The time course of α -galactosidase A activity in sampled tissues indicates that
- 5 administration of DeAd/ α -gal A of vector produces a many fold increase in α -galactosidase A activity in all tested tissues, relative to naive mice, that persists for up to 14 days.

Concurrent with the increase in α -galactosidase A levels in the tested tissues is a significant decrease in GL3 levels in all tissues tested.

Claims

1. A partially deleted adenoviral (DeAd) vector comprising an adenovirus genome from which a majority of adenovirus early genes have been deleted and the major late promoter (MLP) has been replaced by a conditional promoter.
- 5 2. The vector of Claim 1 in which the deleted genes are E1A/E1B, E2, E3, E4, pIX and pIVa2.
3. The vector of Claim 1 in which the conditional promoter is selected from the group consisting of the dimerizer gene control system, the ecdysone gene control system and the tetracycline gene control system.
- 10 4. A DeAd vector producer cell comprising within its genome a majority of adenovirus early genes under control of a conditional promoter.
5. The producer cell of Claim 4 in which the adenoviral early genes are E1A/E1B, E2, E4ORF6, E4ORF6/7, pIX and pIVa2.
6. The producer cell of Claim 4 in which the conditional promoter is
15 selected from the group consisting of the dimerizer gene control system, the ecdysone gene control system and the tetracycline gene control system.
7. A PAV producer cell capable of packaging functional PAV comprising within its genome the majority of adenoviral early genes and the adenoviral major late transcription unit operably linked to a conditional promoter.
- 20 8. The producer cell of Claim 7 wherein the adenoviral early genes are E1A/E1B, E2, E4ORF6, E4ORF6/7, pIX and pIVa2 and the late transcription unit comprises structural virion genes L1 through L5.
9. The producer cell of Claim 7 wherein the conditional promoter is
selected from the group consisting of the dimerizer gene control system, the ecdysone
25 gene control system and the tetracycline gene control system.
10. The vector of Claim 1 wherein the adenovirus is selected from the group consisting of serotypes Ad2, Ad5, Ad6, and Ad17.

11. The vector of Claim 1 wherein the deletion comprises nucleotide 358 of the adenovirus genome through the TATTA box of the adenovirus major late promoter (MLP).

12. The vector of Claim 11 wherein the deletion comprises nucleotides
5 selected from the group consisting of nucleotides 358-6038, 358-6007, 358-6012 and 358-6026 of the adenovirus genome.

13. The vector of Claim 1 further comprising a transgene operably linked to expression control sequences inserted into the adenovirus genome.

14. The vector of Claim 13 wherein the transgene is selected from the
10 group consisting of nucleic acids encoding cystic fibrosis transmembrane conductance regulator (CFTR), human α -galactosidase A, erythropoietin (EPO), factor VIII and factor IX.

15. The vector of Claim 13 wherein the expression control sequences comprise a promoter selected from the group consisting of CMV, PGK and
15 adenovirus E1A promoters.

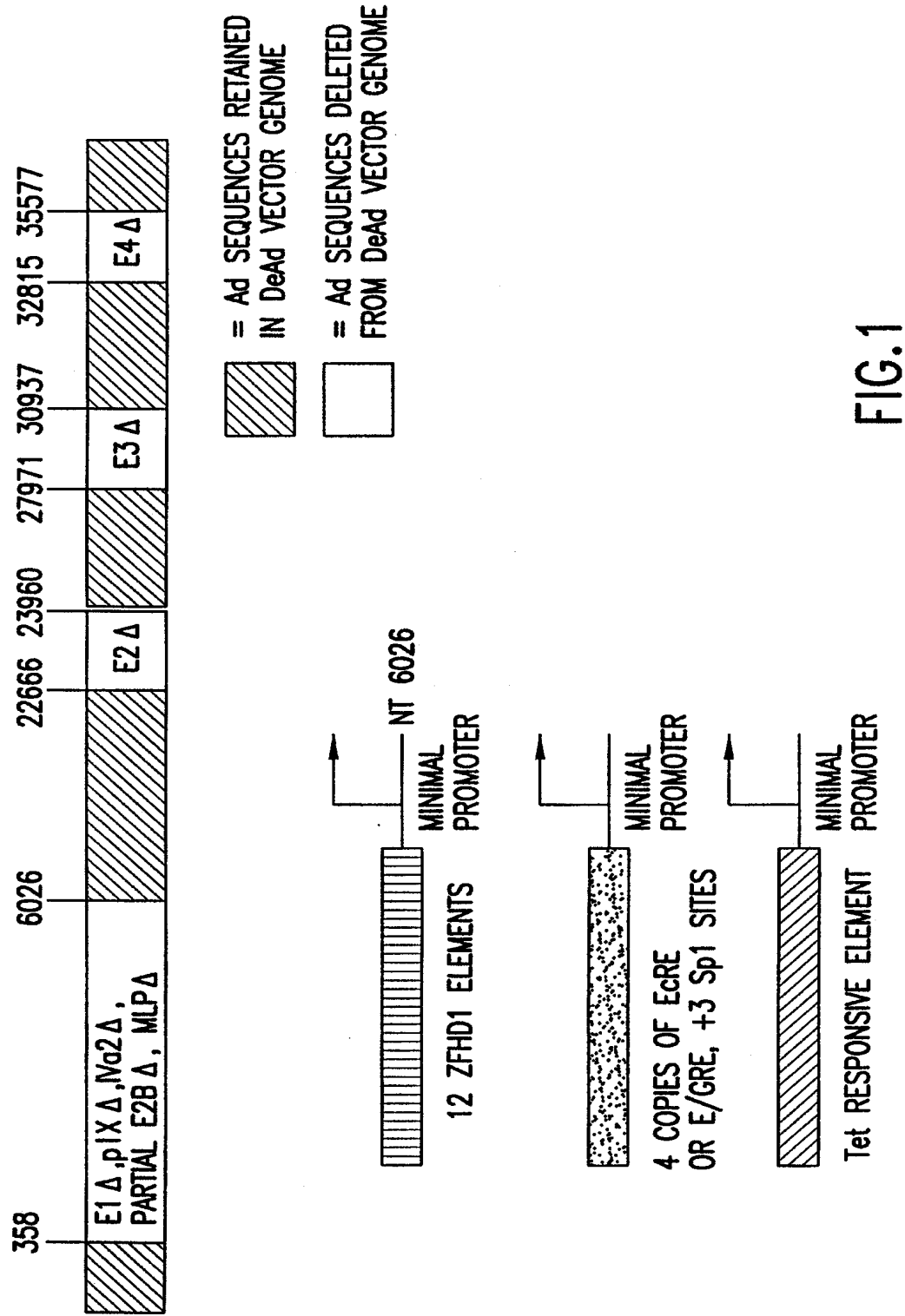
16. The vector of Claim 1 further comprising a deletion of the VA RNA I and VA RNA II genes from the adenovirus genome.

17. A helper virus for production of pseudoadenoviral (PAV) vectors comprising a partially deleted adenoviral (DeAd) vector comprising an adenovirus
20 genome from which a majority of adenovirus early genes and VA RNA genes have been deleted and the major late promoter (MLP) has been replaced by a conditional promoter.

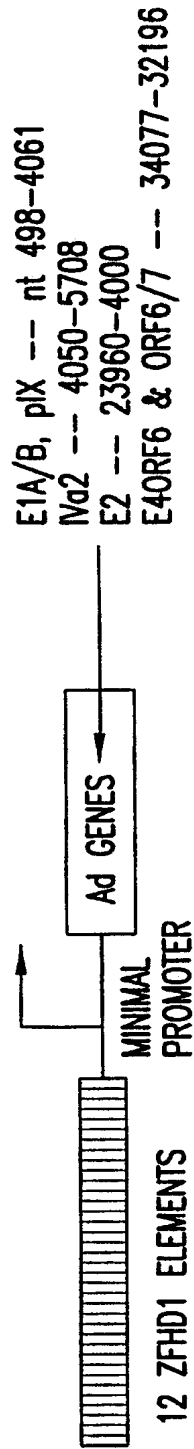
18. The helper virus of Claim 18 in which the deleted genes are E1A/E1B, E2, E3, E4, pIX, pIVa2, VA RNA I and VA RNA II.

25 19. The helper virus of Claim 17 in which the conditional promoter is selected from the group consisting of the dimerizer gene control system, the ecdysone gene control system and the tetracycline gene control system.

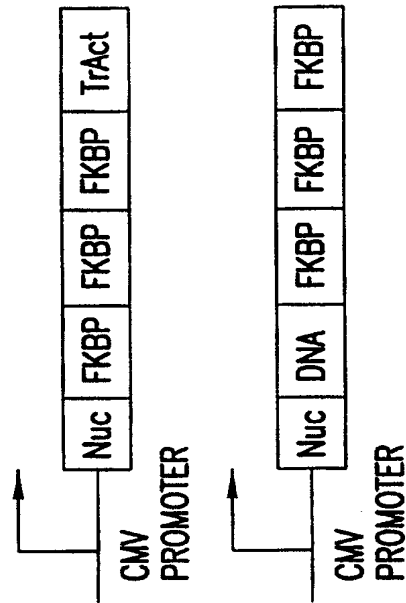
20. The helper virus of Claim 18 wherein the deletion comprises nucleotides 358-6038 and 10600-11030 of the adenovirus genome.



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DIMERIZER GENE CONTROL ELEMENTS STABLY ASSOCIATED WITH THE PRODUCER CELL



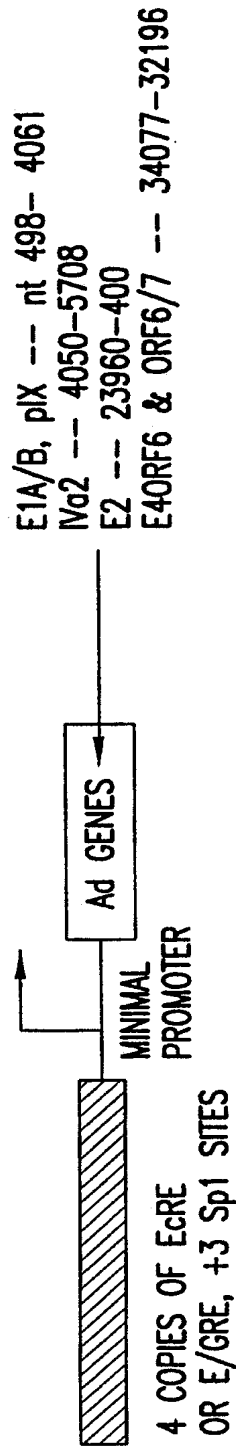
Nuc=NUCLEAR LOCALIZATION SIGNAL
FKBP=THE FKBP12 FK506 BINDING PROTEIN
TrAct=A TRANSCRIPTIONAL ACTIVATOR DOMAIN
DNA=A DNA BINDING DOMAIN

FOR USE WITH FK506-BASED DIMERIZER, THE DNA BINDING DOMAIN AND THE TRANSACTIVATOR DOMAINS ARE BOTH EXPRESSED AS A GENETIC FUSION WITH FKBP.

FOR USE WITH RAPAMYCIN-BASED DIMERIZER, EITHER THE DNA BINDING DOMAIN OR THE TRANSACTIVATOR DOMAIN IS EXPRESSED AS A GENETIC FUSION WITH FRB.

FIG.2

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ECDYSONE GENE CONTROL ELEMENTS STABLY ASSOCIATED
WITH THE PRODUCER CELL

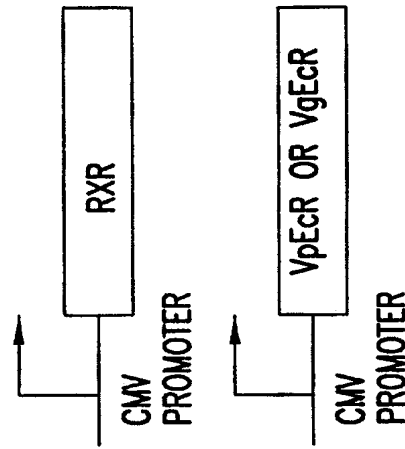
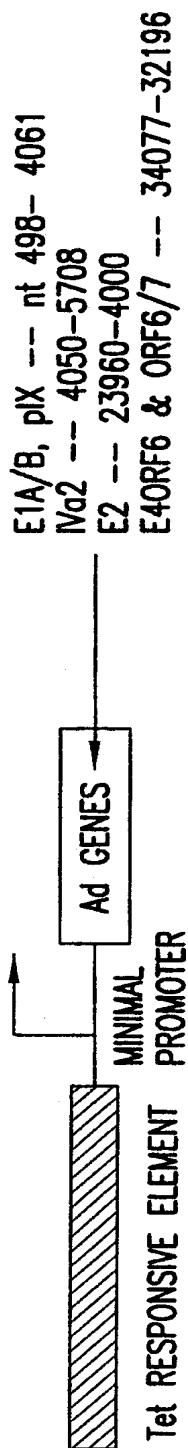


FIG.3



Tet GENE CONTROL ELEMENTS STABLY ASSOCIATED
 WITH THE PRODUCER CELL

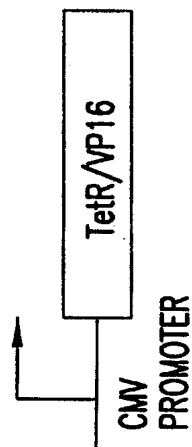
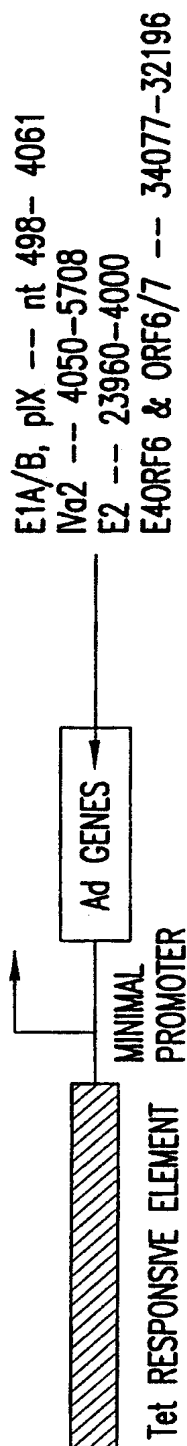


FIG.4



Tet GENE CONTROL ELEMENTS STABLY ASSOCIATED
WITH THE PRODUCER CELL

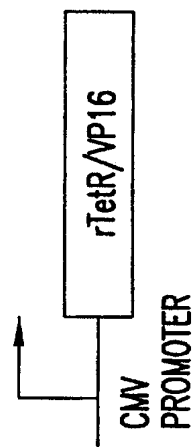
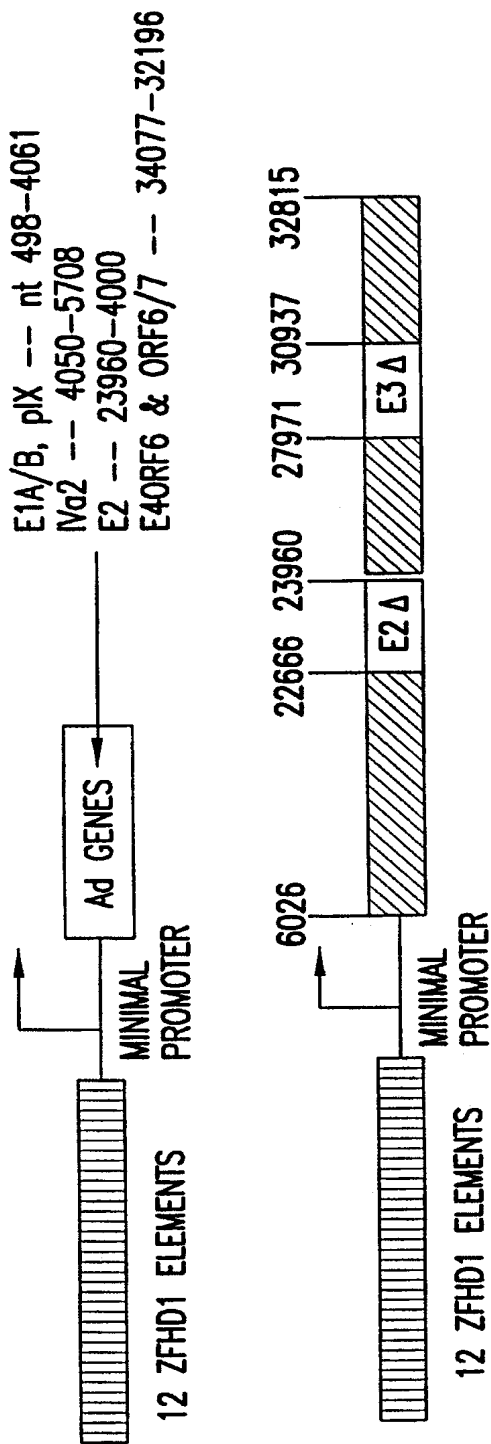
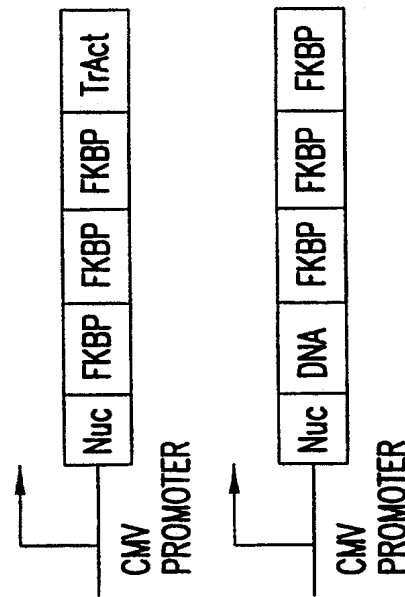


FIG.5



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DIMERIZER GENE CONTROL ELEMENTS STABLY ASSOCIATED WITH THE PRODUCER CELL

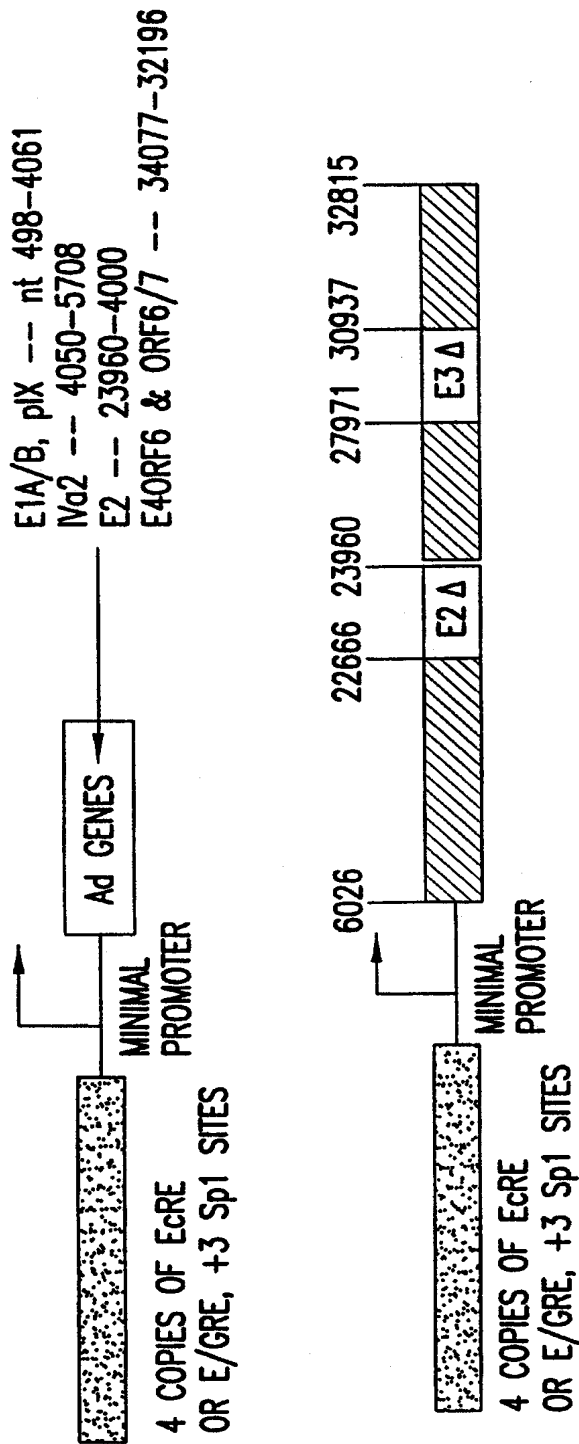


Nuc=NUCLEAR LOCALIZATION SIGNAL
FKBP=THE FKBP12 FK506 BINDING PROTEIN
TrAct=A TRANSCRIPTIONAL ACTIVATOR DOMAIN
DNA=A DNA BINDING DOMAIN

FOR USE WITH FK506-BASED DIMERIZER, THE DNA BINDING DOMAIN AND THE TRANSACTIVATOR DOMAINS ARE BOTH EXPRESSED AS A GENETIC FUSION WITH FKBP.

FOR USE WITH RAPAMYCIN-BASED DIMERIZER, EITHER THE DNA BINDING DOMAIN OR THE TRANSACTIVATOR DOMAIN IS EXPRESSED AS A GENETIC FUSION WITH FRB.

FIG.6



ECDYSONE GENE CONTROL ELEMENTS STABLY ASSOCIATED
WITH THE PRODUCER CELL

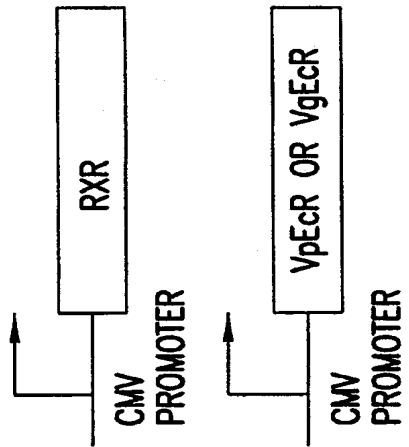
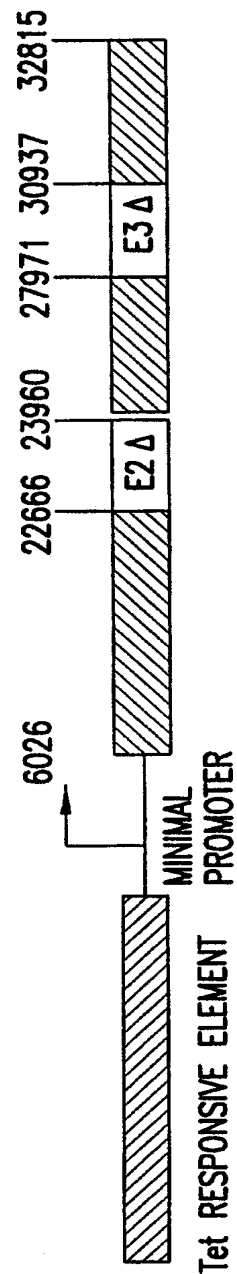
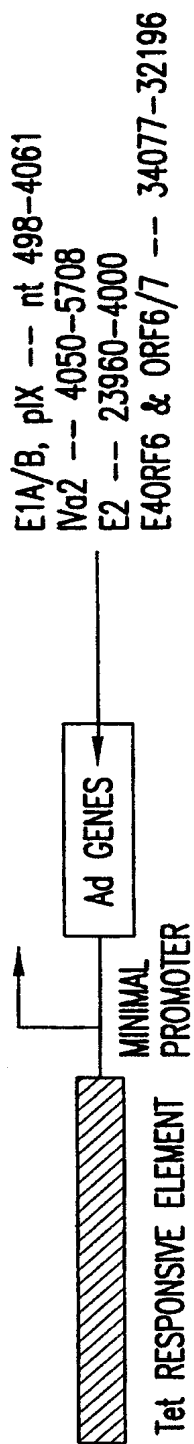


FIG.7



Tet GENE CONTROL ELEMENTS STABLY ASSOCIATED
 WITH THE PRODUCER CELL

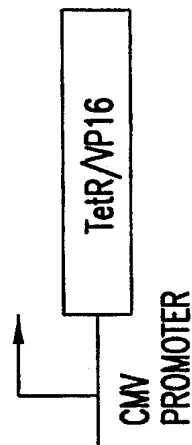
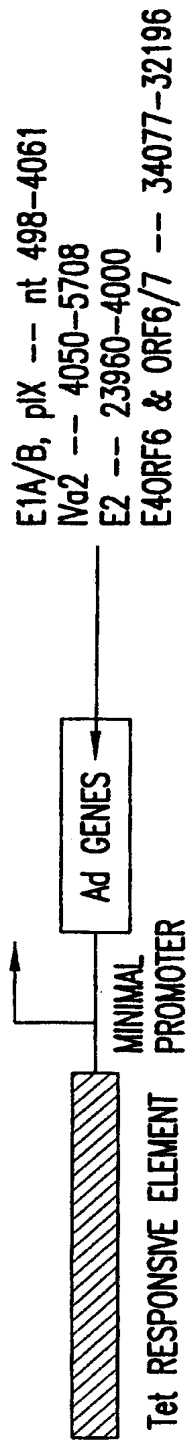
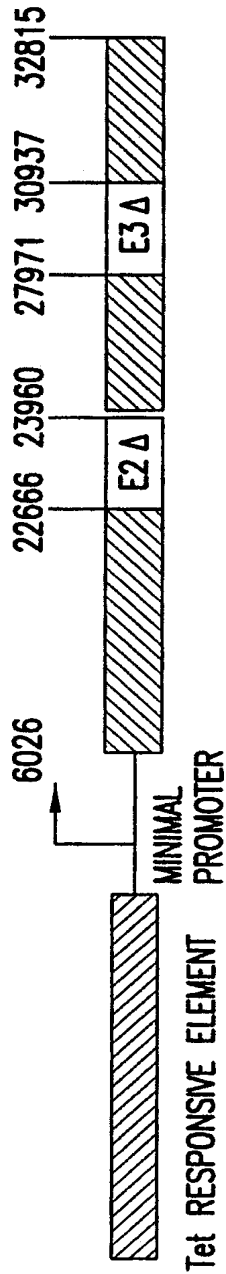


FIG.8



SUBSTITUTE SHEET (RULE 26)



Tet GENE CONTROL ELEMENTS STABLY ASSOCIATED
WITH THE PRODUCER CELL

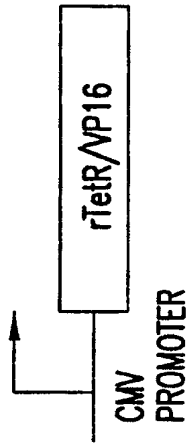


FIG.9

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/09590

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 14061 A (CELL GENESYS INC) 17 May 1996 (1996-05-17)	1,3,4,6, 7,9,10, 13,14
Y	page 5, line 3 - page 7, line 5; table 1 page 19, line 16 - line 22	2,5,8, 11,12, 15-20
Y	MITANI K ET AL: "RESCUE, PROPAGATION, AND PARTIAL PURIFICATION OF A HELPER VIRUS -DEPENDENT ADENOVIRUS VECTOR" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, 1 April 1995 (1995-04-01), pages 3854-3858, XP000619167 ISSN: 0027-8424 abstract; figure 1 page 3857, column 2	2,5,8, 11,12, 16-20

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

20 August 1999

Date of mailing of the international search report

03/09/1999

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Authorized officer

Sprinks, M

INTERNATIONAL SEARCH REPORT

Int. Application No.
PCT/US 99/09590

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	THIMMAPPAYA ET AL.: "Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection" CELL, vol. 31, December 1982 (1982-12), pages 543-551, XP002112814 cited in the application the whole document ---	16-20
A	HEHIR K M ET AL: "MOLECULAR CHARACTERIZATION OF REPLICATION-COMPETENT VARIANTS OF ADENOVIRUS VECTORS AND GENOME MODIFICATIONS TO PREVENT THEIR OCCURRENCE" JOURNAL OF VIROLOGY, vol. 70, no. 12, December 1996 (1996-12), pages 8459-8467, XP002911790 ISSN: 0022-538X the whole document ---	1-20
A	TRIBOULEY ET AL.: "The product of the adenovirus intermediate gene IVa2 is a transcriptional activator of the major late promoter" JOURNAL OF VIROLOGY, vol. 68, no. 7, July 1994 (1994-07), pages 4450-4457, XP002112815 cited in the application the whole document ---	1-20
Y	WO 96 26742 A (HAMMOND H KIRK ;UNIV CALIFORNIA (US); DILLMAN WOLFGANG H (US); GIO) 6 September 1996 (1996-09-06) example 4 -----	15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/09590

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